Hort Innovation

Final Report

Remediation of Soil Contaminated by *Salmonella enterica* to Expedite Plant of Replant of Vegetables

Project leader:

Robyn McConchie

Delivery partner:

University of Sydney

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Remediation of Soil Contaminated by *Salmonella enterica* to Expedite Plant of Replant of Vegetables – VG13039

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Level 8
1 Chifley Square
Sydney NSW 2000

Telephone: (02) 8295 2300

www.horticulture.com.au

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Summary
Salmonella enterica is an important food-borne pathogen. It is the second leading cause of notified food-borne illness in Australia and is of significant concern to consumers, agricultural and food industries and public health agencies. During the past three decades, there has been a rise in the incidence of outbreaks of *S. enterica* associated with the consumption of fresh fruit and vegetables. Contamination of horticultural cropping soils usually occurs through use of raw or uncomposted manures, contaminated irrigation water or incursion of animals in the field. Soils containing *S. enterica* have the potential to contaminate fresh produce through soil contact or through rain or irrigation splash and pose a significant threat to food safety, especially of leafy greens.

This Final Report described the first research done on survival of *S. enterica* in soil contaminated with chicken manure conducted under Australian conditions. The research investigated the effect of soil type, temperature, moisture and presence/absence of chicken manure on survival of this pathogen. This study also examined potential short-term remediation strategies of cover cropping and/or solarisation to reduce levels of *S. enterica* in soil following contamination to allow a quick return to safe crop production.

Our research indicates that *S. enterica* counts decline over time under natural field conditions after a contamination event. However, the rate of decline is significantly slower in clay loam soils, and is reduced by the presence of chicken manure, by soils temperatures less than 37ºC and by the presence of moisture. In the field trial *S. enterica* was detectable up to 100 days after contaminated chicken manure was incorporated into soil. In contrast, populations quickly declined within 4 weeks in sandy soils, at temperatures above 37ºC and in soil without chicken manure as a source of energy.

We also found that solarisation (black plastic covering the soil) may have potential to promote faster die-off of *S. enterica* providing soil temperatures under the plastic have several hours at 37^0C or above.

The use and incorporation of the commercially-available cover crops, Ethopian Mustard, Oilseed Radish and Fumig8tor Sorghum, significantly enriched the soil microbiome after incorporation into the soil but were not effective in this experiment in promoting die-off of *S. enterica* in soil. This may have been due to short growing time (1 month) and uneven cover of the cover crop which limited the amount of biomass and, consequently, biofumigant incorporated in the soil. Further research is required to fully explore the value of cover cropping as a remediation strategy for reclaiming soil contaminated with *S. enterica*. Additional research is needed to determine the amount of biomass and biofumigant levels required in the soil for die-off of *S. enterica* and how quickly this could occur.

The Guideline for Fresh Produce Management (2015) (https://freshproducesafety-anz.com/guidelines/) recommend that untreated manure is not added to soils used for production of short-term crops such as leafy salad greens or herbs. This research will assist growers in assessing the risk and likelihood of food safety outbreaks with *S. enterica* through consideration of on-farm soil characteristics and agronomic practices for remediation to reduce populations of the pathogen in soil after a contamination event using untreated chicken manure.

Keywords
Food safety, vegetable production, abiotic factors, enteric pathogen, food-borne pathogen, *Salmonella* survival

Introduction

Salmonella enterica is an important food-borne pathogen. It is the second leading cause of notified food-borne illness in Australia (OzFoodNet Working Group, 2012) and is of significant concern to consumers, agricultural and food industries and public health agencies. During the past three decades, there has been a rise in the incidence of outbreaks of *S. enterica* associated with the consumption of fresh fruit and vegetables. This worrisome trend is at least partly explained by social changes that have increased the complexity of produce supply chains and their exposure to microbial contamination, growth or recontamination risks. For example, busier lifestyles have promoted the growth of convenience foods, such as 'fresh-cut', 'ready-to-eat', and pre-prepared products, which require high-care facilities and highly controlled transport, distribution and retail environments.

Recent food safety outbreaks in fresh produce have brought the adequacy of current quality assurance systems into question. For example, in 2015 there was a high-profile recall of imported frozen berries due to hepatitis A contamination in Australia. In 2016, there was a recall of 30 prepacked salad products due to *S. enterica* contamination. An official report from Food Standards Australia New Zealand (FSANZ) is not yet available, but the outbreak has been associated with 62 illnesses and two hospitalisations according to media reports (http://www.theage.com.au/victoria/salmonella-outbreak-rises-to-62-cases-in-victoria-from-contaminated-looseleaf-lettuce-20160208-gmod3r.html).

While the sources of food-borne disease outbreaks are investigated by public health and food safety agencies, they can rarely be attributed to specific causes. For fresh produce, food safety may be compromised at critical points throughout the supply chain from the production environment, during processing, transport and handling, in the wholesale and retail environment and through to consumer handling.

In the production environment, animal manure is considered to be one of the major sources of pre-harvest contamination. Faecal matter, whether deliberately used as a soil amendment or dispersed by animal activity, may transfer pathogens to produce by direct contact or by contaminating soil or water, which is subsequently transferred to produce. The use of animal manure-based soil amendments is common practice around the world, particularly where vegetable production occurs in close proximity to intensive livestock production and in organic farming systems that rely on use of organic amendments for crop fertiliser. The benefits are numerous, from improved soil structure, water retention and nutrient profile, to value-addition of an animal waste stream. Soil amendment with manure is safe when the manure is properly composted or treated to control pathogens however problems arise when manures are not managed appropriately.

In this study we examined how environmental factors including soil type, manure amendment, temperature and moisture fluctuation affected the persistence and survival of the pathogen in the soil. We presupposed that a contamination event with *S. enterica* has occurred and explored practical remediation and recovery measures that could be applied to soil to minimize the survival of *S. enterica* and prevent transfer to the edible portion of a harvested crop, in the event of contamination by inadvertent application of improperly or inadequately managed poultry manure and manure composting.

Methodology
Experiment 1: Laboratory study

Objective
This was an exploratory study to investigate the factors and interactions influencing the on-farm component of food safety risk. We investigated the effect of soil type, manure amendment, temperature, and moisture fluctuation on the persistence and survival of the *S. enterica* pathogen in the soil under controlled conditions. This experiment took place prior to the larger field trial.

Experimental design
The study was a full-factorial design comprising five factors: *Salmonella enterica* serovar (inoculum), soil type, manure amendment, temperature and moisture (Table 1). Each treatment combination was applied to 21 replicate sample containers and three replicates were destructively sampled each week for 7 weeks. Triplicate uninoculated control samples, which were subjected to all combinations of environmental factors, were assessed at the start and end of the experiment. See Appendix 1 for additional details of the experimental methods used.

Table 1. Factors tested in the full-factorial experimental design.

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

*The cocktail comprised *S.* Enteritidis, *S.* Infantis, *S.* Montevideo, *S.* Typhimurium and *S.* Zanzibar

Soil preparation and manure amendments
Bulk batches of soil were sourced from The University of Sydney research farms in NSW, Australia. Sandy soil was collected from 'Karalee Farm', Camden, while clay loam was collected from the 'John Bruce Pye Farm', Bringelly. Manure amendment was with aged poultry manure sourced from a local retailer. The soil and manure were sieved through a 2 mm wire mesh then weighed into 120 mL sample containers. Samples were either 100 g soil or 98 g soil mixed with 2 g manure. Properties of the sandy soil and clay loam were determined in the laboratory using standard soil analysis methods and are described in Table 2.

Table 2. Physicochemical properties of soil types.

Parameter	Sandy soil	Clay loam
pH ($CaCl_2$)/pH (water)	5.0/5.8	4.8/5.4
Texture	2.0	2.0
Electrical conductivity (µS)	23.6	124.0
Fine sand:silt:clay (%)	27.0:2.60:5.20	27.0:27.1:35.9
Organic carbon content (%)	3.80	0.80
N:P:K (mg kg^{-1})	98:112:650	14:33:91
Total nitrogen content (%)	0.04	0.37
Bulk density	1.12	1.26

Salmonella cultures
The isolates of *S. enterica* used in this study were a gift from Birling Avian Laboratory (Baiada Poultry Pty Ltd, Bringelly, NSW). The selected serovars represented diverse serotypes that are commonly associated with the Australian poultry system (Table 3). The isolates were maintained on tryptic soy agar (TSA; Becton, Dickinson and Company, USA).

Table 3. *Salmonella enterica* serovars used in this study.

Serovar	Subspecies	Serogroup	Serotype*
Enteritidis	I	D_1	1,9,12:g,m:-
Infantis	I	C_1	6,7,14:r:1,2
Montevideo	I	C_1	6,7,14:g,m,[p],s:[1,2,7]
Sofia	II	B	1,4,12:b:[e,n,x]
Typhimurium	I	B	1,4,[5],12:i:1,2
Zanzibar	I	E1	3,{10}{15}:k:1,2

*Antigenic formulae according to Kaufman-White scheme

Preparation of inocula

The purity of isolates was ensured by re-isolating from overnight cultures (37°C, 18–24 h) grown on xylose lysine deoxycholate agar (XLD; Becton, Dickinson and Company, USA), sub-culturing to nutrient agar (NA; Becton, Dickinson and Company, USA) then confirming the serogroup of single colonies using somatic antisera (Staten Serum Institute, Denmark) in slide agglutination tests. From these plates, five colonies were suspended in phosphate buffer (pH 7.2), spread on TSA plates and incubated overnight (37°C) to generate lawn cultures. The lawn cultures were transferred to phosphate buffer (pH 7.2) and diluted to produce 6 log CFU mL^{-1} liquid inocula. The *Salmonella* cocktail comprised approximately equal concentrations of *S*. Enteritidis, *S*. Infantis, *S*. Montevideo, *S*. Typhimurium and *S*. Zanzibar such that the final total concentration was also 6 log CFU mL^{-1}.

Inoculation

Immediately after preparation, 5 mL inoculum was added to each soil sample excluding the uninoculated controls. The liquid inoculum was kept well-mixed. An additional 5 mL 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 loam. An equivalent volume of water was added to uninoculated controls (i.e. 10 mL). The containers were closed and thoroughly mixed by rotation on a drum-mixer for 20 min.

Temperature and moisture conditions

Containers were incubated at either 5, 21, or 37°C with either the screw-caps firmly closed to maintain the moisture content throughout the study or, to simulate fluctuating moisture, the caps were replaced by gauze to allow evaporation. Incubation temperatures were monitored using min-max thermometers, and did not vary more than ± 1°C during the study. The gross weight of containers was monitored daily and weight loss was used as a guide to maintain the moisture content between predetermined ranges, of 5–15% (w/w) in sandy soil and 10–20% (w/w) in clay loam. For the fluctuating moisture treatment, 10 mL water was added when the gross weight reached the minimum limit, thereby resulting in a periodic variation in moisture that was dependent on the rate of evaporation.

Sample enumeration

Each soil sample (100 g) was weighed into a sterile 400 mL filter bag and manually massaged for 1 min in 100 mL soil extraction medium (0.02 M sodium phosphate with 0.1% Tween 20). The samples were allowed to stand for 15 min and then 5 mL extract was transferred to a falcon tube and used for enumeration. The soil extract (50 µL) was spread onto replicate XLD agar plates using an EasySpiral Automatic Spiral Plater (Interscience, France), incubated (37°C, 18–24 h) and enumerated. Colonies with typical morphology (i.e. red colonies with black centres) were presumptively identified as *S. enterica*. A Scan 500 Automatic Colony Counter (Interscience, France) was used to estimate the plate count and thus calculate the population size of *S. enterica* in the soil sample.

Sample enrichment

Double-strength buffered peptone water (BPW) (95 mL) was added to the remaining 95 mL sample extract, mixed gently and incubated (37°C, 18–24 h). One mL BPW enrichment was mixed with 9 mL tetrathionate broth (TTB) and incubated (42°C, 6 h). One mL TTB enrichment was mixed with 9 mL mBroth and incubated (37°C, 18–24 h). The mBroth enrichment was streaked on XLT4 and CHROMagar Salmonella Plus agar plates and incubated (37°C, 18–24 h). The growth of mauve-coloured colonies, presumptively identified as *S. enterica*, was considered to be positive detection of the pathogen.

Statistical analysis
Enumeration data was the average of plate replicates for each sample. Both the binary survival data and the pathogen population data were analysed in R software using logistic and log-normal models. Soil, manure, temperature, moisture and time after inoculation were treated as fixed effects, while serovar was a random effect in the model. Sample number was also treated as a random effect as samples were prepared and sampled discretely rather than repeatedly. Main effects and pairwise interactions only were included in the model. While higher order interactions could be analysed, pairwise interactions are of greater relevance as they lend themselves to practical interpretation.

Experiment 2: Field trial

Objectives
In this experiment we simulated a natural *Salmonella* contamination scenario in a lettuce crop to explore practical remediation and recovery measures that could be applied to soil to minimise the survival of *S. enterica* (and the potential survival of another soil-borne pathogen, *Listeria monocytogenes*) and prevent transfer to the edible portion of a harvested crop.

Our objectives were to:
(a) determine the optimal low-residue cover crop that enhances die-off of *S. enterica* in contrasting sandy and clay soils,
(b) establish 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, and
(c) assess the potential for increase of *L. monocytogenes* in cover crop-amended soils under field conditions.

Experimental design
The experiment was a split-plot block design with the whole plot treatment being type of cover crop (mustard, radish, sorghum or none) and the split plot treatment being solarisation or no solarisation. Field sites were located at the same two sites from which soils were collected for the laboratory study: 'Karalee Farm', Camden which has sandy soil and 'John Bruce Pye Farm', Bringelly which has clay loam soil.

Preparation of field sites
University field sites were prepared as for commercial lettuce production with four raised beds (1.5 m width) either side of a central bed which was left as a fallow buffer zone. A 0.8 m wide wheel track separated the rows. Treatment plot areas (12 m in length) were marked with stakes along the rows with 2 m and 4 m buffer zones to separate the whole plot treatments.

Preparation of inocula
Rifampicin-resistant *S.* Montevideo was selected by sub-culturing on TSA with incremental rifampicin amendment. Lawn cultures of rif-resistant *S.* Montevideo were prepared on TSARP and transferred to dechlorinated tap water for application to chicken manure pellets. Chicken manure pellets were inoculated twice to achieve a level of contamination of not less than 5 log CFU mL^{-1} (Table 4). Inoculated chicken pellets were applied to the designated treatment plots at the rate of 200 g m^{-2} by hand and incorporated into the top 5 cm of soil by raking (Figure 1).

Table 4. *Salmonella enterica* concentration in chicken manure pellets after inoculation calculated from 100 g manure samples and enumerated on either TSARP or XLDRP plates.

Media	Log CFU g^{-1} (mean ± SD)	
	1st inoculation (7 January 2015)	2nd inoculation (13 January 2015)
TSARP	3.82 ± 0.09	5.18 ± 0.07
XLDRP	3.67 ± 0.01	5.07 ± 0.05

Figure 1. Application of chicken manure pellets containing at least 5 x 10^5 Log CFU g^{-1} rif-resistant *Salmonella* Montevideo at 'Karalee Farm', Camden.

Planting material

Green Oakleaf lettuce seedlings were obtained from a commercial supplier and transplanted into the field using a mechanical transplanter on 16 January 2015. Two rows of seedlings were planted in each bed, excluding the buffer zone, at a spacing of approximately 30 cm (Figure 2). Seedlings that did not survive were replaced by hand the following week. Seedlings were irrigated immediately after transplanting and thereafter as required. Lettuce seedlings were allowed to grow for 4 weeks prior to being ploughed in and incorporated into the soil using a rotary hoe.

Cover crop and solarisation treatments

Following lettuce incorporation, cover crop treatments were applied. The cover crops included 'Terranova' Oilseed Radish (*Raphanus sativus*) and 'Cappuccino' Ethiopian Mustard (*Brassica carinata*) (Seedforce, Shepparton, Victoria, Australia) and were applied by hand at a rate of 10 and 15 kg ha^{-1}, respectively, as recommended for commercial use. 'Fumig8tor' Sorghum seed (Pacific Seeds, Toowoomba, Queensland, Australia) was sown at a rate of 3.1 g m^2 in furrows, as recommended by the supplier. Plots were irrigated immediately after sowing and thereafter as required. Cover crop treatments were ploughed in and incorporated into the soil 35 days after sowing.

Black plastic (6 x 4 m) was applied to control split plots (no cover crop) at the time of cover crop sowing, and to the remaining split-plots after the cover crops were ploughed in (Figure 2). See Appendix 2 for experimental treatment design used for both field sites.

Figure 2. Cover crops growing at 'Karalee Farm' prior to incorporation into the soil.

Weather data and soil conditions

Weather data was obtained from weather stations (Bureau of Meteorology 2015) closest to the field sites (i.e. Camden and Badgerys Creek weather stations). Soil moisture at the field sites was measured each sampling time. Soil temperature was logged at hourly intervals using Thermochron iButtons (OnSolution Pty Ltd, NSW, Australia) at the soil surface and at 5 cm depth at both field sites.

Soil sampling

Soil was sampled for presence of rif-resistant *S. enterica* and *L. monocytogenes* before treatments were initiated and thereafter every week after inoculation for a total period of 91 days. Soil samples were also collected for analysis of phenolics, glucosinolates and microbial diversity. The soil sampling regime and analytical methods are detailed in Appendix 2.

Microbial analysis

Soil samples were extracted as follows: 100 g soil was weighed into a sterile 400 mL bag. The samples were massaged (stomached 1 min at low speed) in 150 mL soil extraction medium (0.02 M Na_3PO_4 with 0.1% Tween 20) and allowed to stand for 15 min before transferring a 1 mL aliquot to a sterile microtube. *Salmonella enterica* in the soil extracts (50 µL) were enumerated after spreading onto replicate XLDRP and TSARP agar plates using an EasySpiral Automatic Spiral Plater (Interscience, France) and incubation (37°C, 18–24 h). Colonies with typical morphology were tentatively identified as *S. enterica*. The plate count and *S. enterica* population in soil was estimated using a Scan 500 Automatic Colony Counter (Interscience, France). When the plate count was below the limit of detection, the remaining soil extract was enriched with 150 mL double-strength buffered peptone water (BPW), mixed gently and incubated (37°C, 18-24 h). A 10 mL aliquot of BPW enrichment was mixed with 90 mL tetrathionate broth (TTB) and incubated (42°C, 6 h). A 10 mL TTB enrichment was then mixed with 90 mL mBroth and incubated (37°C, 18–24 h). The mBroth enrichment was streaked on both XLT4RP and TSARP. Black colonies on the XLT4RP were streaked on CHROMagar Salmonella Plus agar plates. Mauve colonies on CHROMagar were presumed to be *S. enterica*.

The presence of *L. monocytogenes* in soil samples was determined following enrichment only. For this, 25 g soil was massaged (stomached 1 min at low speed) with 225 mL Demi-Fraser broth and incubated (30°C, 24 h). The enrichment was then streaked on CHROMagar Listeria agar plates. Blue-green colonies were presumed to be *L. monocytogenes*.

Microbiome analysis
A subset of the total soil samples collected was used for microbiome analysis. This included a soil sample from each split plot treatment at both sites collected just prior to incorporation of the cover crop (18 March 2015) and about 2 weeks after incorporation (8 April 2015). DNA was extracted from 0.25 g crushed soil sample in a glass bead tube using The PowerLyzer PowerSoil DNA Isolation Kit (Mo BIO Laboratories Inc., California, USA) according to manufacturer protocols. The quality and yield of extracted DNA were determined using a Nanodrop 2000 spectrophotometer, electrophoresis and fluorimetry. According to the DNA concentration, the samples were normalised to 5 ng μl^{-1} with an elution buffer.

Dual-barcoded PCR amplicons were prepared using a two-step PCR protocol (PCR1 and PCR2). PCR1 was added and then specific marker genes were amplified for determination of the targeted microbial community. The primers were the 16S rRNA (I1-I4) gene for bacteria and ITS2 (I1-I4) region for fungi. The enzyme used was Phusion DNA polymerase. All plates included negative and positive controls (sample with 16S marker). PCR2 added index sequences and Illumina sequencing adaptors to the gene-specific product obtained on PCR1, in pre-mixed 96-well microplates for either bacterial or fungal taxa.

The PCR products were pooled depending on yield and then purified using the Bioline Isolate II PCR and Gel kit (Bioline (Aust.) Pty Ltd, NSW, Australia), following manufacturer protocols. DNA concentrations were measured by nanodrop and fluorimetry Quant-iT PicoGreen dsDNA assay (Invitrogen, Ltd, UK). The purified DNA products were sequenced by Micromon (Monash University) on an Illumina MiSeq using V3 chemistry.

Analysis of foliar phenolics
The relative total content of phenols in foliage of the three cover crops (mustard, radish and sorghum) was estimated using a modified Folin-Ciocalteu assay (Cicco et al. 2009). Freeze-dried plant material (0.05 g) was vortex-mixed with 5 mL 80% methanol (aq) then extracted by ultrasonication (40°C, 10 min). The extract was centrifuged (6000 rpm, 10 min) and the supernatant used for the assay. The reaction mixture comprised 100 µL gallic acid standard or sample extract, 100 µL Folin-Ciocalteu reagent, and 800 µL 5% sodium carbonate in a 2-mL microtube. The microtube was incubated in a hot water bath (20 min, 40°C) then transferred to an ice bath to cool rapidly. An aliquot of 200 µL reaction mixture was transferred to triplicate wells of a 96-well microplate. A gallic acid standard curve (0–160 µg mL^{-1} in 20 µg mL^{-1} increments) was used to determine the total phenols content of the sample extracts in gallic acid equivalent units.

Statistical analysis
Population data for *S. enterica* was analysed using a General Linear Mixed Model (GLMM) in GenStat 16^{th} edition software (VSN International, UK). Pathogen population counts from the Camden (sandy soil) site declined rapidly and were below the limit of detection by 28 January 2015, just 14 days after the contaminated chicken manure pellets were incorporated into the soil. This was prior to application of any cover crop treatments, so only the Bringelly (clay loam) site data were statistically analysed. The GLMM assumed a Poisson distribution and used a logarithmic link function. The effects of time, whole plot (cover crop), and split plot (solarisation) factors were analysed, with a component of random variation attributed to time and sample. Effects were considered significant where $P<0.05$.

The microbiome sequencing data were analysed as follows. The quality statistics of the sequencing run were assessed using the NGS QC toolkit application (Patel and Jain 2012). The sequence reads (R1 and R2) were joined using the PEAR read joiner using default settings (Zhang et al. 2014). Demultiplexing (sorting of sequences by barcode), quality filtering, OTU picking and diversity analyses were carried out using the QIIME analysis pipeline (Caporaso et al. 2010).

Outputs

Output 1: Data from experiments

Experiment 1: Laboratory study
Under controlled laboratory conditions, this experiment investigated the influence of *Salmonella enterica* serovar, soil type (sandy and clay loam), temperature (5, 21 and 37°C) and chicken manure (0 or 2% w/w) on survival of *Salmonella* serovars (*S*. Enteritidis, *S*. Montevideo, *S*. Sofia and a *Salmonella* cocktail). Serovar survival over time followed a similar pattern. Therefore, only the results for the *Salmonella* cocktail are presented below.

Soil type, temperature and presence/absence of manure significantly influenced survival. Over time, the likelihood of detection of *S*. *enterica* declined but this decline was hastened in sandy soil, at higher temperatures and in the absence of manure. For sandy soils incubated at 21°C without addition of manure, survival of *S*. *enterica* declined rapidly to non-detectable limits 21 days after inoculation (Figure 3A). In clay soils, *S*. *enterica* declined fairly rapidly over the first 4 weeks but then remained at 10^3–10^4 CFU g^{-1} soil (Figure 3A). Soil amended with chicken manure significantly increased the survival of *S*. *enterica* over time in clay soils (Figure 3B) and more so in clay loam than in sandy soil (data not shown).

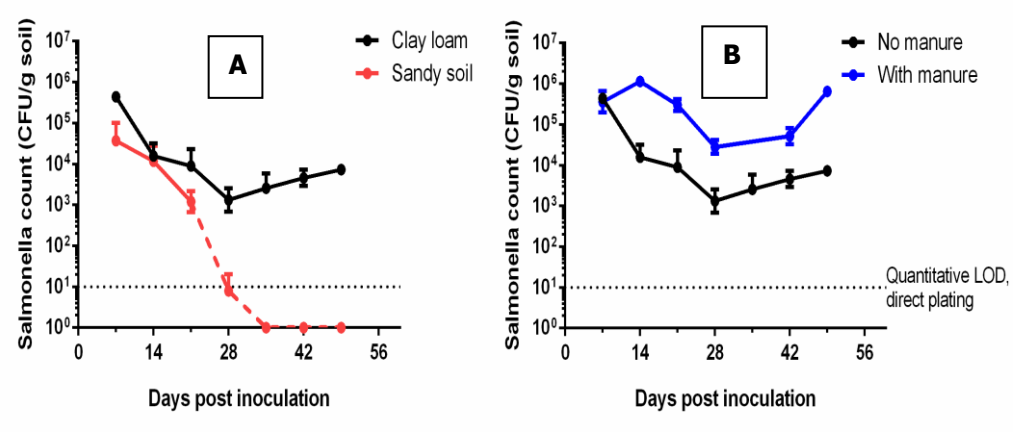

Figure 3. A *Salmonella* count (CFU g^{-1} soil, mean ± SD) in clay and sandy soil incubated at 21°C for 49 days (n = 3); **B** *Salmonella* count (CFU g^{-1} soil, mean ± SD) in clay soil with 2% (w/w) poultry manure added and incubated at 21°C for 49 days (n = 3).

Survival of *S*. *enterica* was significantly influenced by incubation temperature (Figure 4A). At 37°C in clay soils with no added manure, *S*. *enterica* declined rapidly to below detection limit within 4 weeks. At 5 and 21°C, survival declined slightly but remained around 10^4 CFU g^{-1} soil (Figure 4A). However, the adverse effects of high temperature, particularly 37°C, on likelihood of *Salmonella* detection were mitigated by soil amendment with chicken manure (Figure 4B). At all temperatures in clay soils, survival of *S*. *enterica* was promoted by the presence of manure.

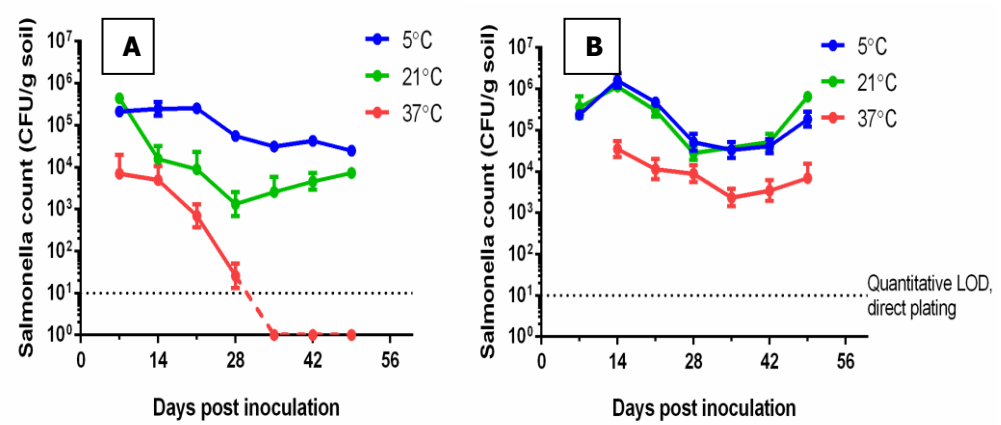

Figure 4. A *Salmonella* count (CFU g^{-1} soil, mean ± SD) in clay soil incubated at 5, 21 or 37°C for 49 days (n = 3); **B** *Salmonella* count (CFU g^{-1} soil, mean ± SD) in clay soil with 2% (w/w) poultry manure added and incubated at 5, 21 or 37°C for 49 days (n = 3).

The importance of the environmental factors and interactions was also examined more quantitatively by comparing data at specific time points during the study. Two weeks after inoculation (Table 5), *S. enterica* recovery in sandy soil was below the detection limit in most treatments, but was slightly higher than the initial inoculum density in clay loam (5.7 $\log_{10}$ CFU g^{-1}) under the least stressful conditions of 5°C and constant moisture. As the temperature of the clay loam was increased to 21°C, recovery of *S. enterica* underwent a 2 log reduction, while a further increase to 37°C brought an additional 0.7 log reduction in *S. enterica* when other factors were equal.

The effect of temperature on persistence of *S. enterica* was relatively consistent in clay loam at constant moisture. For example, one or more log reductions associated with higher temperature were also observed 4 and 6 weeks after inoculation (Tables 6 and 7). The trend was not consistent across all treatment combinations due to significant interactions of temperature with other environmental factors. The additional stress of fluctuating moisture tended to exacerbate the decline of *S. enterica*. In both clay loam and sandy soil, amendment with poultry manure greatly prolonged persistence of *S. enterica*. Comparing populations in the putatively more stressful 37°C-fluctuating moisture treatments, manure amendment improved recovery of *S. enterica* in clay loam nearly three-fold (3.8 compared to 1.3 $\log_{10}$ CFU g^{-1}). Manure amendment of sandy soil enabled survival of *Salmonella* (3.1 $\log_{10}$ CFU g^{-1}) which was below the detection limit in sandy soil alone (Table 5).

Table 5. Recovery of *Salmonella* cocktail at 2 weeks after commencement of incubations. Treatments include soil type, manure amendment, temperature and moisture (n = 3).

	Concentration ($\log_{10}$ CFU g^{-1})					
Treatment	Constant moisture			Fluctuating moisture		
Soil + amendment	5°C	21°C	37°C	5°C	21°C	37°C
Clay loam	5.4 ± 0.1	3.4 ± 0.9	2.7 ± 0.5	4.9 ± 0.6	1.6 ± 1.6	1.3 ± 1.2
Clay loam + manure	5.7 ± 0.1	5.4 ± 0.2	3.9 ± 0.5	4.8 ± 0.4	4.1 ± 0.2	3.8 ± 0.6
Sandy soil	<LOD*	2.8 ± 0.7	<LOD	4.6 ± 0.0	<LOD	<LOD
Sandy soil + manure	4.0 ± 0.3	2.3 ± 0.8	4.1 ± 0.1	2.6 ± 2.2	2.8 ± 1.2	3.1 ± 0.3

*<LOD: below limit of detection

Table 6. Recovery of *Salmonella* cocktail at 4 weeks after commencement of incubations. Treatments include soil type, manure amendment, temperature and moisture (n = 3).

	Concentration (log_{10} CFU g^{-1})					
Treatment	Constant moisture			Fluctuating moisture		
Soil + amendment	5°C	21°C	37°C	5°C	21°C	37°C
Clay loam	4.5 ± 0.1	3.1 ± 0.6	<LOD*	2.4 ± 0.1	1.0 ± 0.9	<LOD
Clay loam + manure	3.1 ± 2.7	<LOD	3.2 ± 0.5	2.6 ± 0.7	3.3 ± 0.8	2.4 ± 2.1
Sandy soil	3.9 ± 0.1	<LOD	<LOD	<LOD	<LOD	<LOD
Sandy soil + manure	4.7 ± 0.1	4.7 ± 0.0	4.3 ± 0.2	<LOD	<LOD	<LOD

*<LOD: below limit of detection

Table 7. Recovery of *Salmonella* cocktail after 6 weeks after commencement of incubations. Treatments include soil type, manure amendment, temperature and moisture (n = 3

	Concentration (log_{10} CFU g^{-1})					
Treatment	Constant moisture			Fluctuating moisture		
Soil + amendment	5°C	21°C	37°C	5°C	21°C	37°C
Clay loam	4.4 ± 0.1	3.8 ± 0.1	<LOD*	1.6 ± 1.6	1.2 ± 1.2	<LOD
Clay loam + manure	5.2 ± 0.2	5.8 ± 0.2	2.4 ± 2.2	3.0 ± 1.3	4.8 ± 0.7	5.7 ± 0.1
Sandy soil	1.0 ± 0.9	<LOD	<LOD	<LOD	<LOD	<LOD
Sandy soil + manure	<LOD	1.0 ± 1.7	1.1 ± 0.9	<LOD	<LOD	<LOD

<LOD: below limit of detection

Experiment 2: Field trial

The sequence of events in the field trial was as follows. Manure inoculated with *S. enterica* was applied at commercial rates to both sandy and clay loam soils at the University farms and planted with lettuce seedlings at commercial spacing. After 4 weeks of growth, the lettuce crop was incorporated into the soil and cover crops were sown by seed and allowed to grow for 35 days. Cover crops were ploughed in and covered with black plastic to ensure maximum incorporation of biofumigant into the soil. Soil samples were taken from each treatment plot at weekly intervals and analysed for survival of *S. enterica*.

For clay soil under the control (fallow) treatment, *S. enterica* declined rapidly in the first 4 weeks (Figure 5) which coincided with hot summer daily temperatures (30–40°C) for that period. Around mid-March the rate of decline of *S. enterica* slowed to a level of 10^2 CFU g^{-1} soil by day 91 post-inoculation. The application of black plastic on day 35 significantly hastened the decline of *S. enterica* until day 84 where it was below the level of detection (Figure 5). In contrast, *S. enterica* could still be detected in the control (fallow) treatment on day 84 post-inoculation.

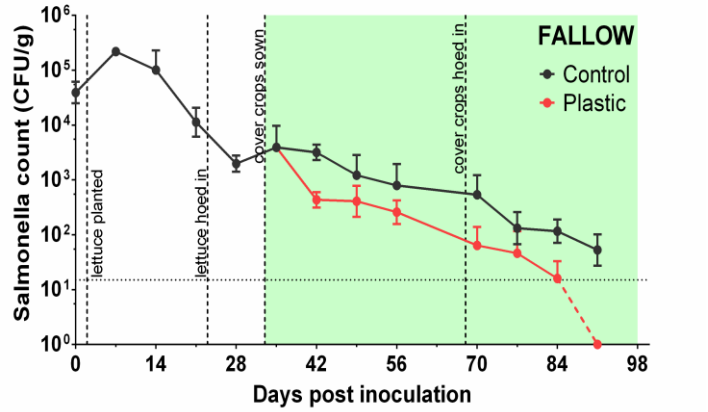

Figure 5. *Salmonella* count (CFU g^{-1} soil, mean ± SD, n = 3) in clay soil sampled weekly under sequential treatments of addition of inoculated manure (days 0–2), planting and growing lettuce seedlings (days 2–23), ploughing in of lettuce crop and resting (days 24–33), and either left fallow or covered with black plastic (days 33–91).

Overall there was no difference among the cover crop treatments in hastening decline of *S. enterica* (Figure 6). All treatments including the control, showed a similar rate of decline from day 33 onwards.

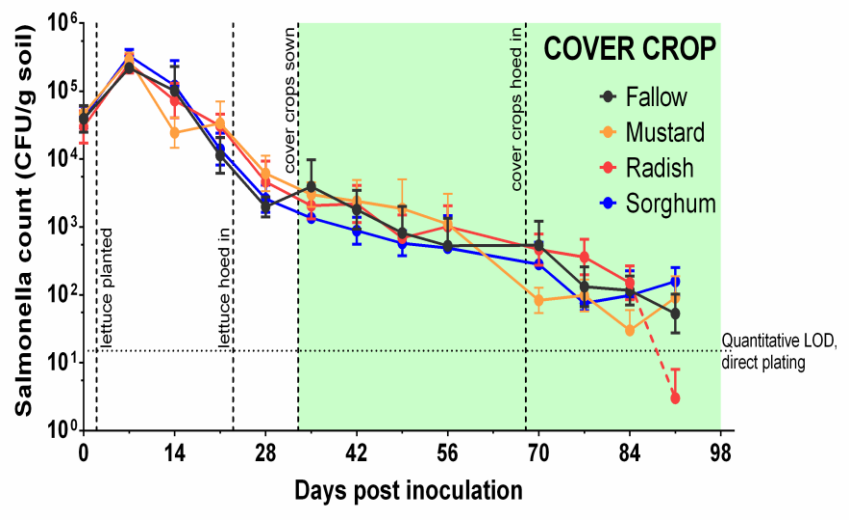

Figure 6. *Salmonella* count (CFU g^{-1} soil, mean ± SD, n = 3) in clay soil sampled weekly under sequential treatments of addition of inoculated manure (days 0–2), planting and growing lettuce seedlings (days 2–23), ploughing in of lettuce crop and resting (days 24–33), sowing and growing cover crops (days 33–67) and ploughing in cover crops (days 68–91).

After the 'Fumig8tor' Sorghum cover crop was incorporated into the soil, the rate of decline of *S. enterica* was significantly increased by the application of black plastic to below the level of detection by day 75 (Figure 7). For the 'Fumig8tor' Sorghum cover crop treatment without solarisation, *S. enterica* remained at detectable levels at around 2 log CFU g^{-1} soil.

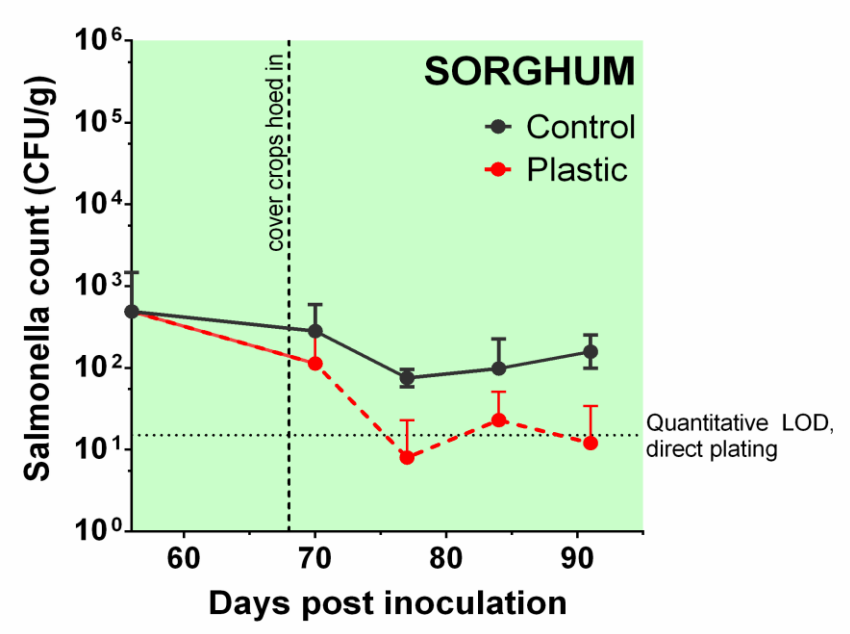

Figure 7. *Salmonella* count (CFU g^{-1} soil, mean ± SD, n = 3) in clay soil sampled weekly from days 56–91 post-inoculation with *Salmonella enterica* and sown with 'Fumig8tor' Sorghum cover crop, ploughed in on day 68 and either left fallow (control) or covered with black plastic for 1 week.

Presence of phenolics and glucosinolates in foliage of cover crops

The cover crop treatments did not significantly expedite die-off of *S. enterica* compared to the control (fallow), as was hypothesised. Rather than dismiss the usefulness of cover crops at this stage, we suggest that parameters around cover crop management to maximise biofumigant efficacy needs to be determined prior to repeating the field trial. During the progress of the field trial, we observed that the growth of the cover crop was highly variable and sparse in some areas. Overhead photographs of the field plots were taken and a crude groundcover index estimated using image manipulation software (Vectorworks 2015 SP1, distributed by OzCAD in Australia).

Considering the four replicate whole plots for each cover crop, for the site with sandy soil, the best coverage was for the mustard treatment (63–85%), followed by radish (43–79%), while sorghum had the least coverage (19–58%). For the site with clay soil, the best coverage was again for mustard (75–87%), followed by sorghum (37–78%) and radish (32–56%). Figure 8 demonstrates the range of growth of cover crop at the latter site.

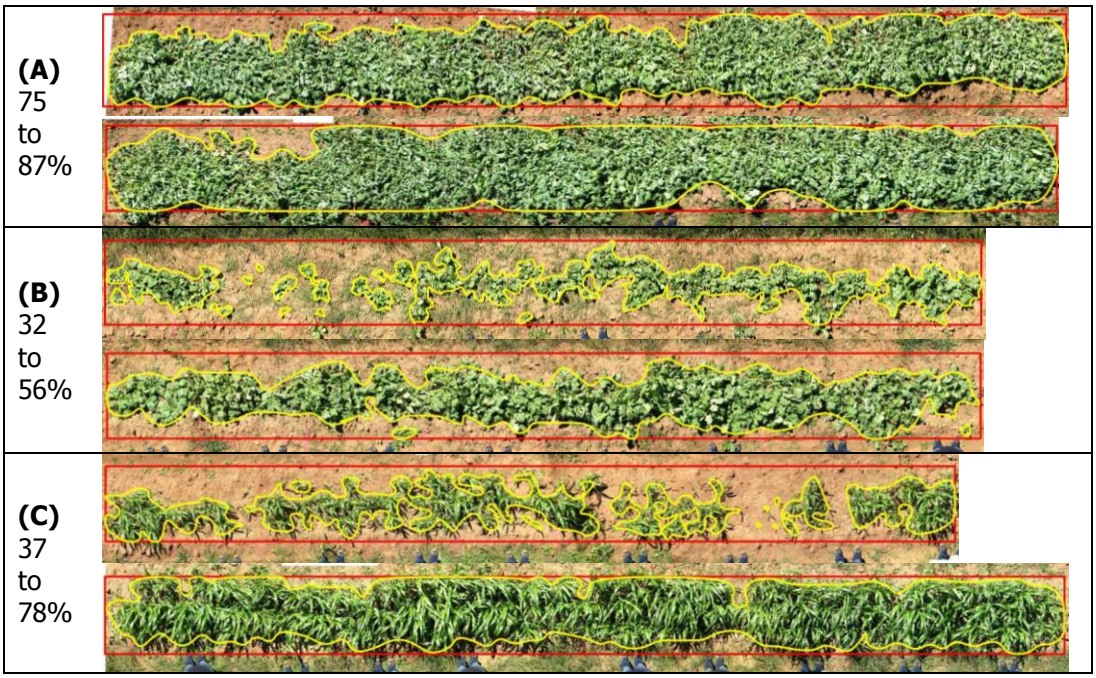

Figure 8. Overhead photographs of field plots at 'John Bruce Pye Farm', Bringelly, demonstrating the range of groundcover achieved for whole plots of **A** 'Cappuccino' Ethiopian Mustard; **B** 'Terranova' Oilseed Radish; and **C** 'Fumig8tor' Sorghum.

Given the poor efficacy of the cover crop treatments in our field trial, the phenolic composition and glucosinolate levels in the soil were not determined. Instead, total phenolics content and glucosinolates in the cover crop samples were analysed to estimate the relative potential contribution of the plants. Total phenolics were estimated using a modified Folin-Ciocalteu assay (Table 8).

Table 8. Total phenolic content of cover crop plants in gallic acid equivalent concentration (n = 3).

Cover crop	Total phenol content (µg GAE g dw)
'Cappuccino' Ethiopian Mustard	5119 ± 1188
'Terranova' Oilseed Radish	6117± 837
'Fumig8tor' Sorghum	7519 ± 1750

Weather conditions and soil temperature

The field trial began in mid-summer on 5 January 2015. Conditions were relatively mild for this region with daily maximum temperatures in January ranging from 30–35°C (Table 9, Figures 9 and 10) with several overcast days. Nevertheless, the population of *S. enterica* at the site with sandy soil decreased to below the limit of detection by 14 days after application of contaminated manure in the field. This corresponded with the laboratory study where high temperature interacted significantly with soil type, leading to much poorer survival in sandy soil than clay loam. In the field trial, there was a rapid die-off of *S. enterica* despite manure amendment and irrigation.

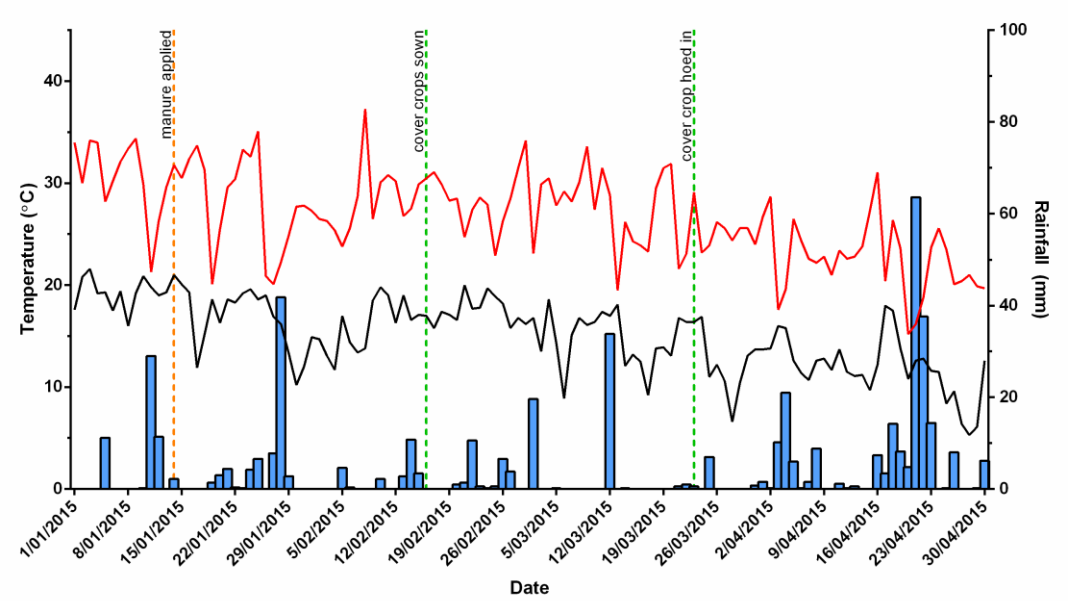

Figure 9. Daily weather conditions at 'Karalee Farm', Camden during the field trial (data from the Camden station, Bureau of Meteorology). The black line is minimum daily temperature (°C); the red line is maximum daily temperature (°C); and the blue bar is daily precipitation (mm).

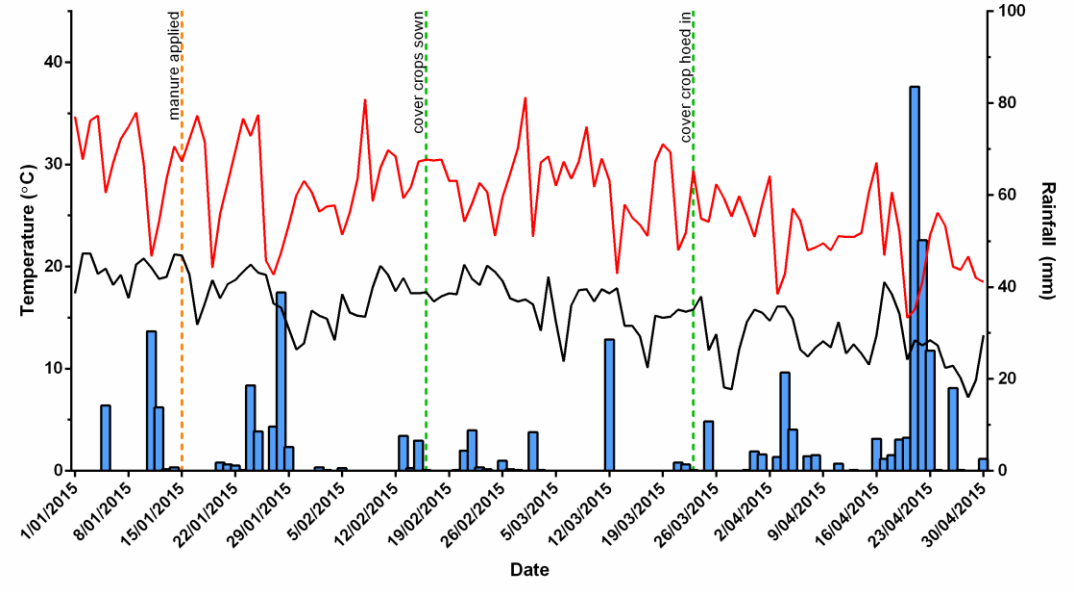

Figure 10. Daily weather conditions at 'John Bruce Pye Farm', Bringelly during the field trial (data from the Badgery's station, Creek Bureau of Meteorology). The black line is minimum daily temperature (°C); the red line is maximum daily temperature (°C); and the blue bar is daily precipitation (mm).

Table 9. Mean maximum and minimum daily temperature and rainfall at the two sites ('Karalee Farm', Camden (Karalee) and 'John Bruce Pye Farm', Bringelly (Pye) during the field trial.

Month	Minimum temperature (°C)		Maximum temperature (°C)		Total rainfall (mm)	
	Karalee	Pye	Karalee	Pye	Karalee	Pye
January	17.9	18.3	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

Black plastic was for used solarisation in this trial rather than clear plastic as this is the commercial practice in Australia. The application of plastic increased the temperature at the soil surface and at 5 cm depth compared to the control (Figures 11 and 12).

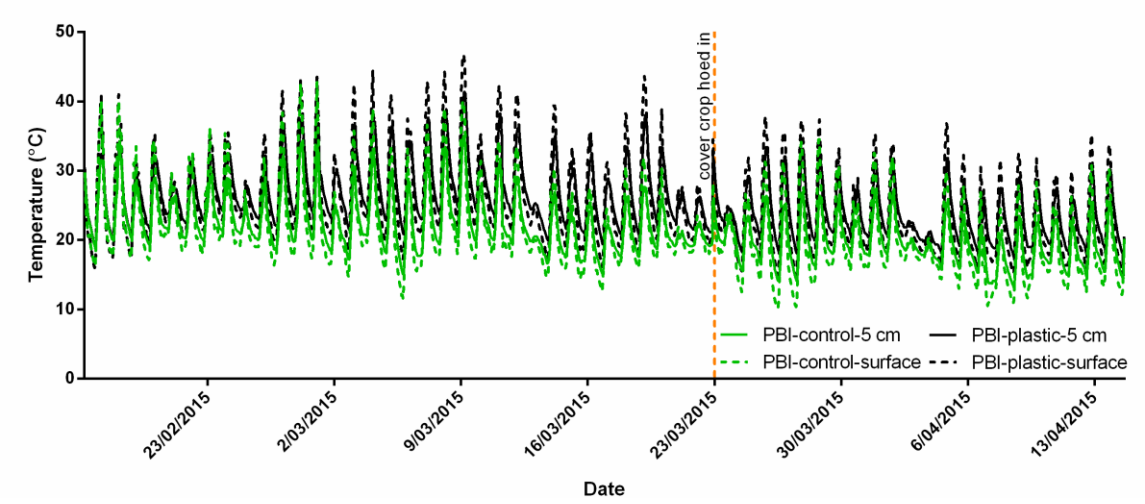

Figure 11. Effect of solarisation using black plastic on soil temperature at the surface and at 5 cm depth at 'Karalee Farm', Camden, measured from 16 February–15 April 2015 (n = 4).

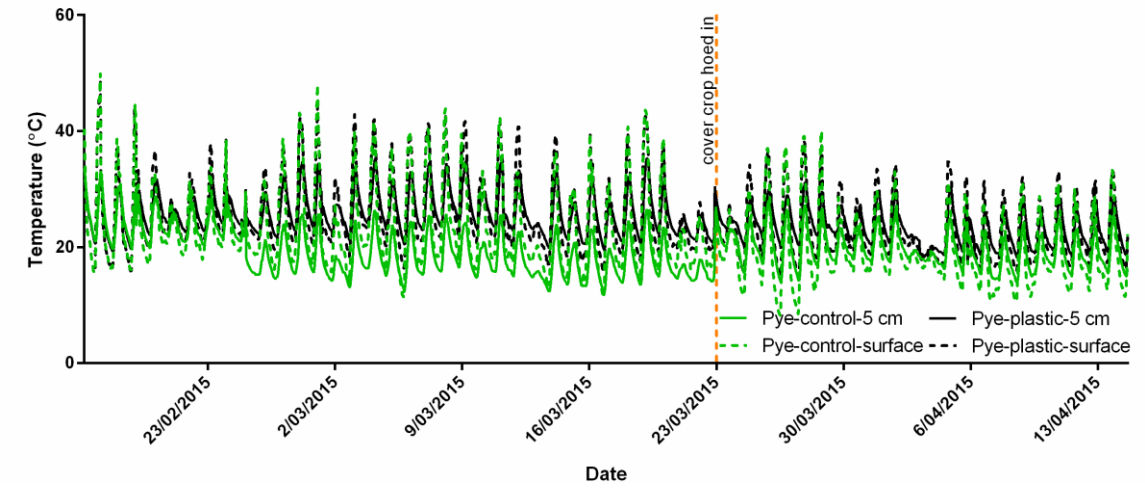

Figure 12. Effect of solarisation using black plastic on soil temperature at the surface and at 5 cm depth at 'John Bruce Pye Farm', Bringelly, measured from 16 February–15 April 2015 (n = 4).

Solarisation significantly increased the average daily minimum and mean and maximum temperatures calculated over the entire field trial at both trial sites (Table 10). Solarisation was expected to expedite die-off of *S. enterica* by increasing the duration of periods of time over 37°C (and preferably even higher). However, the number of hours over 37 and 40°C (Tables 11–14) suggests that solarisation was only likely to work for the fallow-control plots, wherein plastic was installed in late summer (16 February 2015). For the cover crop-solarisation treatments, the plastic was installed in autumn (23 March 2015) and did little to increase soil temperature. However, it is possible that the treatment is still beneficial as the plastic may help to seal in the soil and boost biofumigation effects from incorporation of the cover crop.

Table 10. Difference in daily minimum, mean and maximum temperatures among solarisation and control treatments throughout the field trial at the two sites: 'Karalee Farm', Camden (Karalee) and 'John Bruce Pye Farm', Bringelly (Pye). **Highly significant ($P<0.001$) using paired t-test.

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

Table 11. Soil temperatures in fallow-control solarisation plots at 'Karalee' Farm, Camden (installed 16 February 2015). T denotes temperature; Ctrl denotes Control.

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	
	Plastic	Ctrl	Plastic	Ctrl	Plastic	Ctrl	Plastic	Ctrl
1 week (16/02–23/02)	6	4	2	2	0	0	0	0
2 weeks (16/02–2/03)	20	14	9	5	3	0	0	0
3 weeks (16/02–9/03)	53	22	27	7	18	0	4	0
4 weeks (16/02–16/03)	67	22	33	7	20	0	4	0
Duration (16/02–15/04)	81	22	38	7	23	0	4	0

Table 12. Soil temperatures in fallow-control solarisation plots at 'John Pye' Farm, Bringelly. (installed 16 February 2015). T denotes temperature; Ctrl denotes Control.

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	
	Plastic	Ctrl	Plastic	Ctrl	Plastic	Ctrl	Plastic	Ctrl
1 week (16/02–23/02)	19	17	11	13	0	0	0	0
2 weeks (16/02–2/03)	32	31	18	21	0	0	0	0
3 weeks (16/02–9/03)	62	49	33	28	0	0	0	0
4 weeks (16/02–16/03)	75	54	37	30	0	0	0	0
Duration (16/02–15/04)	92	75	41	36	0	0	0	0

Table 13. Soil temperatures in cover crop-solarisation plots at 'Karalee' Farm, Camden (installed 23 March 2015). T denotes temperature; Ctrl denotes Control.

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	
	Plastic	Ctrl	Plastic	Ctrl	Plastic	Ctrl	Plastic	Ctrl
1 week 23/03–30/03))	4	0	0	0	0	0	0	0
2 weeks (23/03–6/04)	5	0	0	0	0	0	0	0
3 weeks (23/03–13/04)	5	0	0	0	0	0	0	0

Table 14. Soil temperatures in cover crop-solarisation plots at 'John Pye' Farm, Bringelly (installed 23 March 2015). T denotes temperature; Ctrl denotes Control.

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	
	Plastic	Ctrl	Plastic	Ctrl	Plastic	Ctrl	Plastic	Ctrl
1 week (23/03–30/03)	6	10	0	1	0	0	0	0
2 weeks (23/03–6/04)	6	10	0	1	0	0	0	0
3 weeks (23/03–13/04)	6	10	0	1	0	0	0	0

Microbiome analysis

There was a significant difference in microbial richness of soil among bacterial and fungal communities (Figure 13). The QIIME analysis specifies the processed taxonomical results in operational taxonomic units (OUT). The Chao 1 richness estimator (from the QIIME analysis) shows a bacterial taxonomic diversity ~1500 units at 400 sequences per sample while fungal diversity had ~130 units at equal sequencing. Bacteria are considered to be the most abundant and diverse microorganisms in soil with approximately 10^3 to 10^7 bacterial species per individual soil sample. Besides fungi, archaea, and viruses are also numerically abundant, usually in lower numbers than bacteria (Fierer et al. 2007).

The three cover crops and the Control (Figure 14A) presented similar OTU richness results in alpha diversity analysis. Sorghum had greater richness for the bacterial community and mustard and sorghum had greater richness for the fungal community. Radish had a pattern similar to the Control treatment in both communities. Prior studies have demonstrated that addition of biofumigant plant biomass to soil as root exudates, crop tissue or oilseed meal has the potential to stimulate and alter microbial community structure, composition and diversity. However, any changes depend on a variety of factors and it is difficult to determine patterns according to specific phylogenetic taxa (Hollister et al. 2013). Mustard Oilseed and sorghum residues have been shown to alter bacterial and fungal community abundance and structure (Hollister et al. 2013).

Bacterial richness was higher in sandy soil compared clay loam (Figure 14B). This differentiation was evident from a low number of sequences per sample in the rarefaction curve. In contrast, clay loam had greater fungal richness than sandy soil (Figure 14B). This pattern corresponds to findings from by Frey et al. (1999) such that bacteria generally inhabit soil pore spaces where they are physically protected from desiccation and rewetting events. Microbial habitat depends on soil texture and may confer protection to bacteria and enhance survival. In contrast, fungal hyphae are generally found on the exterior of soil aggregates (Strickland and Rousk 2010).

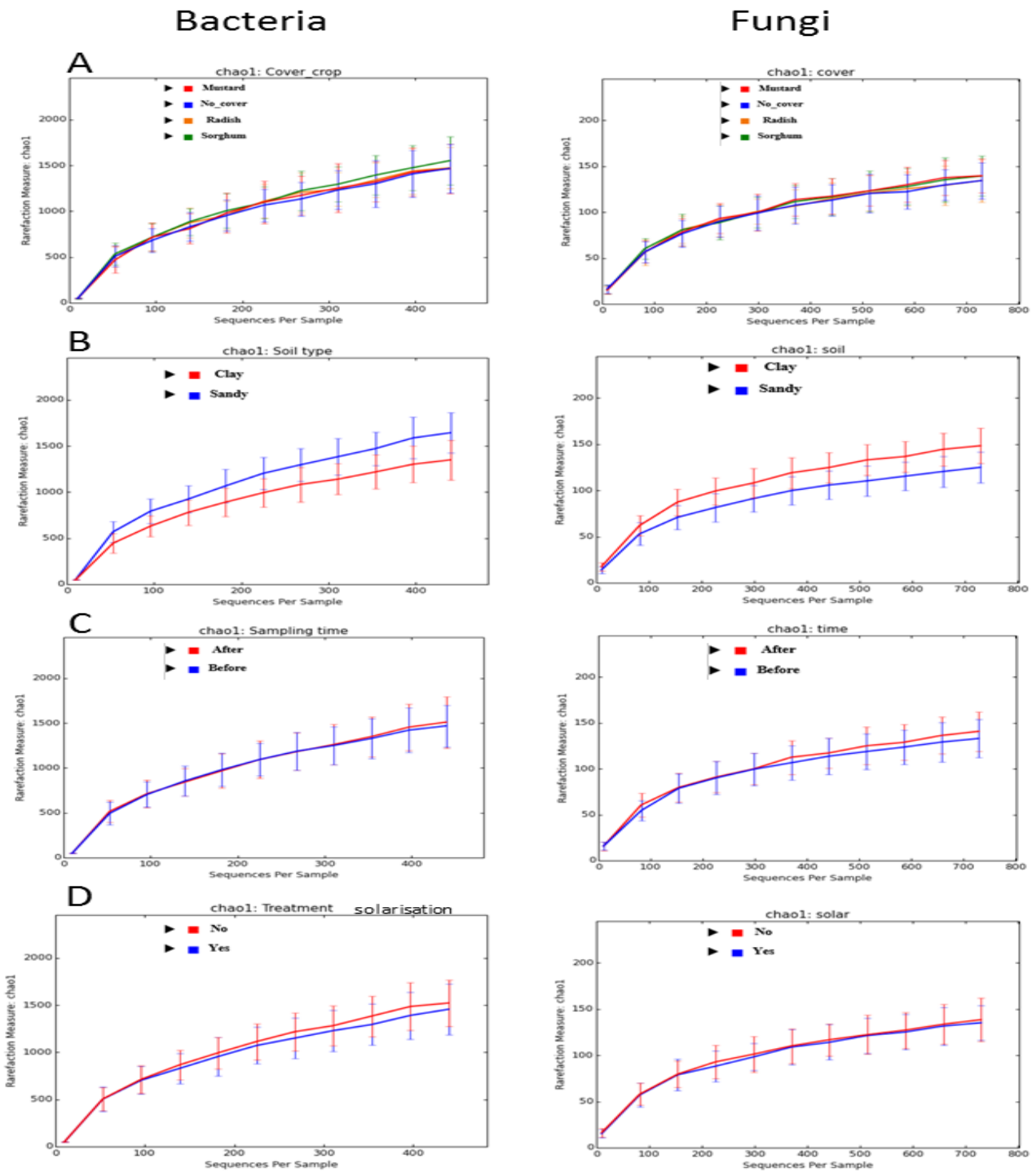

Figure 14. Rarefaction curves showing the richness of soil bacterial and fungal communities in four treatments. **A** Cover crop; **B** soil type; **C** sampling time; **D** soil solarisation. Chao 1 index was the species richness estimator used in this analysis of alpha diversity.

The rarefaction curves also showed that fungal and bacterial OTU richness increased slightly after incorporation (sampling time) of biofumigant cover crops (Figure 14C). The incorporation of carbon-source substrates such as plant tissue has been shown to cause detectable shifts in soil microbial

community characteristics. These changes are greater and generally additive when cover crops are combined with other treatments such as solarisation (Bernard et al. 2012). Shifts in the microbial communities due to biofumigation with Brassica crops may be a response to the incorporation of fresh and decomposable organic matter into the soil (Omirou et al. 2011).

The main mechanism of action in soil solarisation is direct thermal inactivation of microbes (Culman et al. 2006). The field trial revealed only a minor effect on the soil microbiome (Figure 14D). However, it was a slightly higher value on the soil bacterial OTUs richness than the fungal richness. Various studies including Gelsomino and Cacco (2006) have demonstrated that the changes induced in the physical, chemical and biological characteristics of the soil by solarisation usually increase the functioning and diversity of the soil bacterial community, This occurs in soil treated with solarisation only, and in combination with other treatments (Gelsomino and Cacco 2006). However, reports in fungal communities have been more variable (Culman et al. 2006).

The data obtained from the alpha diversity QIIME analysis displayed minor variations, both in bacterial and fungal communities, with respect to average OTU content. Phylogenetic composition was influenced by treatments. Among the main phyla of bacteria, Actinobacteria was enriched in sandy soil while Proteobacteria increased in clay loam soil and Bacteroidetes was enriched in both soil types (Figure 15). Actinobacteria, Bacteroidetes and Proteobacteria increased the average OTU content for the cover crops treatments in most cases. Actinobacteria was the only phylum that had a tendency to increase with the effect of the solarisation treatment mostly in sandy soil.

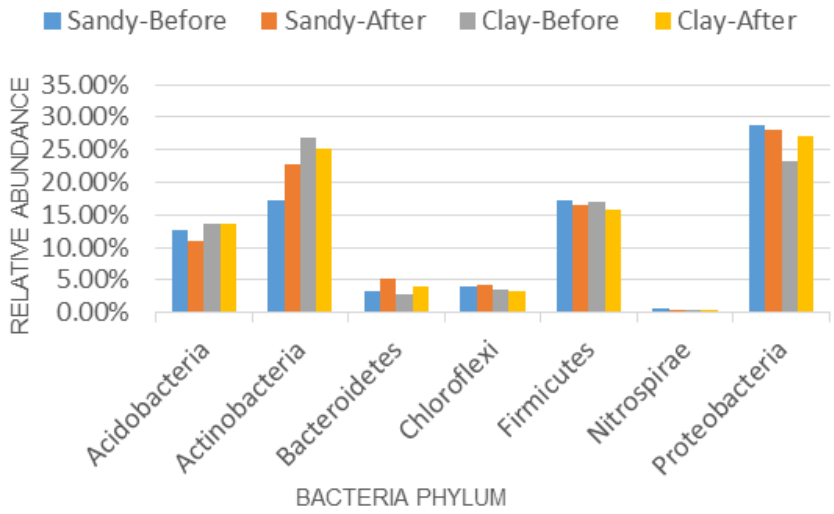

Figure 15. Relative abundance (%) of the main bacterial phyla due to soil type and sampling time.

The taxa associated with the fungal community showed a negligible but similar tendency due to soil type. Ascomycota slightly increased, while Basidiomycota and Zygomycota decreased in sandy and clay loam soil (Figure 16). With respect to cover crop, changes in fungal communities in clay loam were more evident than in sandy soil. Fungi in the phylum Ascomycota decreased with the incorporation of sorghum and mustard, and those in the Basidiomycota only decreased after radish was incorporated into both soil types. OTU content indicting fungi in the Zygomycota increased when both radish and sorghum was incorporated into sandy soil.

For the solarisation treatment, the OTU content of fungi in the phylum Ascomycota was augmented and was reduced for Basidiomycota in all treatments. Overall, the highest OTU content for specific

genera of fungi was for *Fusarium* (19.2%), followed by *Penicillium* (8.3%) and *Aspergillus* (3.2%) which all belong to the phylum Ascomycota (Campaniello et al. 2010).

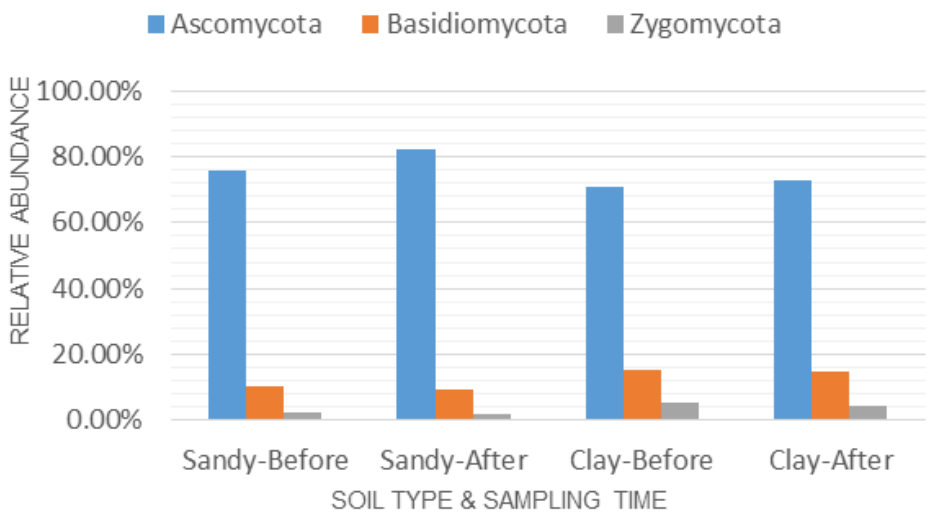

Figure 16. Relative abundance (%) of the main fungal phyla due to soil type and sampling time.

Output 2: Literature review on odour management of chicken manure

In addition to the experimental component of this project, HIA requested compilation of a desk review on odour management of chicken manure. This manuscript has been prepared and undergone several edits and will now be submitted for publication in a peer reviewed journal by the end of June 2016 (not appended due to imminent publishing).

Output 3: Draft fact sheet on 'Odour management of chicken manure' (Appendix 3)

A draft of an extension fact sheet has been prepared for use by farmers. This sheet will be formatted and provided to AusVeg. It will also be posted on the Fresh Produce Safety Centre Website to access a wide grower audience.

Output 4: Draft fact sheet on 'Effects of biofumigants and solarisation on soil microbial communities' (Appendix 4)

A draft of an extension fact sheet has been prepared to inform farmers on the benefits of cover crops in promoting microbiome diversity. This sheet will be provided to AusVeg. It will also be posted on the Fresh Produce Safety Centre Website to access a wide grower audience.

Output 5: Poster prepared for the 29th International Horticulture Congress in Brisbane 17–22 August 2014 (Appendix 5)

Title: 'Remediation of soil contaminated by *Salmonella enterica* to expedite plant or replant of vegetables.'

Output 6: Communication/extension activities

2014

1. US collaboration: Dr Kim-Yen Phan-Thien from the University of Sydney visited Dr Trevor Suslow, our collaborator and lead researcher, at University of California, Davis (UC Davis) from 17–24 May 2014. This travel was fully funded by the University of Sydney. The aim of the trip was to compare methodologies for both the laboratory and field components of the project used in the US and Australia. This was to ensure the validity of comparing results from two well-separated locations. Dr Phan-Thien spent time assisting with the field trial implementation (Figure 17) and laboratory analyses.

2. Centre for Produce Safety symposium: Dr Kim-Yen Phan-Thien attended the Centre for Produce Safety (CPS) Symposium in Newport from 24–27 June 2014. This travel was fully funded by the University of Sydney. Dr Phan-Thien benefitted greatly from presentations from other researchers on food safety. In addition, this was a valuable experience as we were required to present at the CPS Symposium in 2015. Dr Phan-Thien was able to meet with Dr Suslow again to share progress on the project.

Figure 17. Dr Phan-Thien at the field site with Dr Suslow at University of California, Davis in May 2014.

2015

1. US collaboration: Dr Trevor Suslow, our collaborator and lead researcher at UC Davis visited Australia 16–20 March 2015. This travel was funded by UC Davis and the Center for Produce Safety. The aim of the trip was to compare results for both the laboratory and field components of the project and to ensure that similar methods were being used in the US and Australia. While in Australia, Dr Suslow gave seminars at the University of Sydney and at the Future Apples Workshop in Orange on 18 March 2015.

2. US CPC and UC Davis collaboration: Dr Robyn McConchie, Dr Kim-Yen Phan-Thien and Dr Tina Bell were invited to the Center for Produce Safety conference in Atlanta 22–25 June 2015 to present preliminary finding of joint UC Davis/University of Sydney project (Appendix 1). The travel was wholly funded by the University of Sydney. Dr Phan Thien was profiled as a Young Researcher at the conference. Prior to the conference a promotional article on the joint project was published by the CPS (Appendix 7).

3. US CPC and UC Davis collaboration: The Director of the CPS visited Australia from 10–14 August 2015 to review project progress. Ms Fernandez-Fenaroli indicated she was very pleased with the way the collaboration had brought together the FPSC, CPS and researchers to share and produce research of high quality. While in Australia, Ms Fernandez-Fenaroli also attended the annual Australian Institute of Food Science Technology (AIFST) convention (funded by the University of Sydney) and gave an address at the annual Fresh Produce Safety Centre (FPSC) conference (12 August 2015).

4. Invited speaker AIFST Microbiology conference: Dr Robyn McConchie was invited to speak about the establishment and role of the FPSC and her current research projects which included a short overview of this project (VG13039). This was an international conference held over two days and had an estimated audience of 100 attendees (Appendix 8).

5. Fresh Produce Safety conference: Dr McConchie was invited to present an update on this project. The audience for this talk was over 150 people (Appendix 9).

2016

1. Completion of project for Masters in Agricultural Science. Ms Luz Stella Gonzalez Rubio successfully completed her research (microbial community analysis) and graduated in May 2016.

2. **Pending completion of PhD** Ms Mulatua Halu Haifa is expected to complete her thesis in August 2016.

Outcomes

1. Laboratory study outcomes

This section outlines the impact of key environmental factors, namely, soil type, manure amendment, temperature and moisture fluctuation, and their interactions on the probability of sustaining a pathogenic population of *S. enterica* in the soil. The effects of abiotic and biotic factors on pathogen population dynamics is an important topic in applied microbiological research. There has been substantial work to characterise factors affecting bacterial growth in media and specific model food systems, generally with a view to predictive modelling or determining thermal inactivation conditions. The aim of this research was to investigate how such interactions play out in the soil environment, with a view to determining relevance for the agricultural production context, particularly vegetables.

Our results confirmed that the persistence of populations of *S. enterica* (i.e. microbial count) and likelihood of survival (i.e. detection) was significantly influenced by soil type, manure amendment, temperature and moisture fluctuation. Of greater interest perhaps, were significant interactions between soil type × manure, soil type × moisture, temperature × manure and temperature × moisture.

Temperature

The importance of temperature to bacterial growth is well established. There is a direct relationship between temperature and growth rate of *Salmonella* within the growth range of about 7–42°C, other factors held equal (Fehlhaber and Krüger 1998). This is borne out in certain studies of model foods including beef mince (Mackey and Kerridge 1988), chicken (Juneja et al. 2007) and lettuce (Koseki and Isobe 2005) where higher storage temperature increased *Salmonella* growth within the tested range. However, the effect of temperature can be confounded by other factors such as pH, water availability and other variables.

Our study demonstrated that sustained exposure to high temperature (37°C) significantly reduced the likelihood of pathogen survival over the 6 week course of the experiment and had significantly reduced *S. enterica* population by 2 weeks after inoculation. However, the effect of temperature was confounded by an interaction with manure amendment, which not only increased the likelihood of detection of *S. enterica*, but also increased the average size of the population recovered, regardless of temperature. On the other hand, wet-dry moisture fluctuations significantly reduced the recovery of *S. enterica*, overriding the enhancing effect of low temperature on the pathogen.

Soil type

Soil type is known to be an important factor affecting survival food-borne pathogens. High clay content tends to improve pathogen persistence, for example, *S. enterica* and *E. coli* persisted longer in silty clay loam than loamy sand (Natvig et al. 2002). However, the relationship between soil texture (as defined by sand:silt:clay composition) and pathogen survival is not always consistent due to the influence of other factors and interactions (Erickson et al. 2013). Research has been conducted to evaluate the importance of specific soil parameters. For example, in a study of how cattle feeding regime impacted the persistence of *E. coli* O157:H7 and *S.* Typhimurium in sandy and loamy soils due to effects on manure amendment, pH and fibre content were identified to be highly significant (Franz et al. 2005).

Clay minerals alter the physicochemical properties of soil, including cation exchange capacity and surface area, which have a differential impact on pathogen survival. How clay content influences bacterial survival is not fully understood, but is thought to include increased buffering capacity, moisture retention and nutrient availability and small particle size that offer protection from predators, parasites, desiccation and exposure to UV and toxins (Brennan et al. 2014). Other soil parameters that influence pathogen populations include pH and electrical conductivity. Specifically, *S. enterica* and *E. coli* O157:H7 populations declined more rapidly in acidic soils than neutral or

alkaline soils, while dissolved salts are suggested to interfere in ion transport and enzyme activities, also leading to reduced pathogen survival (Erickson et al. 2013). Differences in microbial diversity and composition may also explain the variability in pathogen dynamics in different soils.

Chicken manure

Chicken manure is regarded to be one of the most valuable animal wastes in agricultural production due to its high nutrient content and relatively low cost. However, it contains a wide variety of pathogens including those commonly associated with foodborne disease outbreaks. The most prevalent pathogens isolated from chicken manure are *Salmonella* and *Campylobacter* (Chen and Jiang 2014). Composting is a common technique for pathogen control, as well as improving spreadability and stability of the product for application. Temperatures in well-managed compost heaps should reach 55–65°C, well above the thermal death points of mesophilic pathogens such as *Salmonella*. A number of studies have verified that composting is an effective method for eliminating food-borne pathogens in manures (Chen and Jiang 2014). There have also been a number of studies reporting the persistence of pathogens during composting such that the requisite time-temperature combination for pathogen elimination is not met (Chen and Jiang 2014). The thermodynamics within composting mixtures is affected by a range of factors including composition, cumulative heat, moisture, oxygen and pH (Erickson et al. 2014).

In our study we tested how the presence of chicken manure affects persistence and survival of *S. enterica*. Manure amendment enhanced recovery of *S. enterica* over the course of the experiment, but there were important interactions with soil type and temperature that counteracted the effects of sandy soil and high temperature, which otherwise tended to reduce pathogen persistence. This may be due to the availability of additional nutrients, which is suggested to be a mechanism for prolonged persistence of *Salmonella* in almond orchard soils where dropped nuts and hulls leach nutrients to the soil (Danyluk et al. 2008).

2. Field study outcomes

Soil contamination with human pathogens represents a major hurdle in the fresh produce supply chain because of the lack of mitigation strategies to eliminate persistent contamination. 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 this experiment, we selected a combination of cover crops and agronomic practices, such as solarisation, that are currently used in Australia, to determine their effect on *S. enterica* survival in soils contaminated *via* chicken manure. The target remediation treatments, alone and in combination, included three cover crops and solarisation.

Manure and irrigation water are the main sources of *Salmonella* contamination in soil and horticultural produce. The heterogeneity of the soil greatly influences survival of *Salmonella.* As shown in the laboratory experiment in this report persistence of *S. enterica* is dependent on factors such as moisture, soil type, temperature and energy and nutrient sources such as manure. Currently, manure application is considered to be the most common route by which *Salmonella* is introduced to the soil and has been shown to survive in manure-amended soils for up to 332 days (Jacobsen and Bech 2012).

The soil microbial community has been widely recognised for its importance in regulating soil processes and control of plant and human pathogens. Studies have shown that *Salmonella* survival in soil is influenced by the competitive effects from pre-existing microbial communities. For example in one study, the *Salmonella* population decreased due to competitive effects from the extant microbial community in the soil when introduced with manure (Jacobsen and Bech 2012). Soil microbial communities are influenced by temperature, soil type, plant species and genotype, crop rotations, green manures and cover crops (Mazzola 2004). Their pathogenic suppression is the product of complex changes in the soil microbial community characteristics (Larkin et al. 2010). Soil suppressiveness is the capacity of a soil ecosystem to limit the development of particular soil-borne

pathogen and can be encouraged against a particular pathogen with specific management practices.

Biofumigation describes the suppression of soil pests and diseases by incorporation of bioactive plant tissues into soil. The allelopathic effect of mustard, sorghum and radish cover crops for weed suppression and soil-borne pathogens has been well documented. These plants secrete a number of biochemical compounds as root exudates that possess antimicrobial characteristics and have been regarded as effective against intestinal pathogens when extracted from other plant (Survay et al. 2012). Glucosinolates are considered to be biologically inactive, however their degradation products have toxic effects on fungi and bacteria (Brader et al. 2006). For example, 1-methoxy-3-indolylmethyl (1-MIM) glucosinolate, contained in many *Brassica* vegetables, is strongly mutagenic in *Salmonella typhimurium* TA100 when activated by myrosinase (Wilson et al. 2013).

Soil solarisation is another agronomic practice that has demonstrated positive results in the control of soil pathogens. However, contrasting effects have been reported on the fate of soil microbial populations. Changes in the soil chemical characteristics caused by increased temperatures, severely alter the density and activity of microbial communities (Gelsomino and Cacco 2006; Bonanomi et al. 2008).

Soil type
In the field trial, survival of *S. enterica* was significantly affected by soil type. Survival of *S. enterica* in sandy soils decreased rapidly within a few days after application of chicken manure and remained low or below the limit of detection for the duration of the field trial (98 days). In contrast, survival of *S. enterica* in the clay soil declined more slowly only reaching the limit of detection around day 100. This supports our findings in the laboratory and confirms that clay soils tend to promote the survival of *S. enterica*. Over time, the level of *Salmonella* declined for all treatments in the clay loam indicating that populations naturally decline after a contamination event.

Cover crop
None of the cover crops tested in this experiment were effective in hastening the decline of *S. enterica* in the soil. The rate of decline was similar for all three cover crop treatments as well as the control. A number of factors may have contributed to the lack of suppressive activity by the cover crops against *S. enterica*. The cover crops were sown in autumn when the growing conditions were ideal, however the rate of growth and eventual biomass at the time of ploughing in 4 weeks after sowing, was variable and patchy. This may have led to low levels of biomass incorporation of cover crop and consequently biofumigants, into the soil, thus reducing the effectiveness in suppression of *S. enterica*.

Another important factor that may have influenced efficacy of the cover crops in reducing *S. enterica* populations was the management incorporation of the cover crop into the soil. In the field trial, the cover crop treatments were ploughed in with rotary hoe in a single pass. However this method left a lot of intact plant material. The biofumigant effect requires the breakdown of cellular tissue and release of phytochemicals at a concentration that harm pathogens. Parameters that affect the rate of biomass decomposition, such as particle size, temperature and moisture are likely to be critical and need to be determined for a reliable response.

Solarisation
In the laboratory experiment, die-off of *S. enterica* was significantly increased by temperatures over 37°C. Therefore it was expected that the field solarisation treatment would also increase die-off. In the field, the black plastic solarisation treatment was applied to the fallow treatment (no cover crop) in mid-February. In the following month, 92 and 41 hr were recorded above 37ºC and 40ºC at the soil surface under the plastic, respectively. In this treatment, solarisation did hasten die-off of *S. enterica* compared to the control, reaching levels below the limit of detection at day 84. For the cover crop treatments, solarisation was applied after the cover crop was ploughed in, around 4 weeks later in mid-March, and very few hours were recorded for temperatures at 37°C or above. It

is not surprising therefore that there was no significant in *S. enterica* die-off for two of the three cover crop treatments after solarisation was applied. The exception was for sorghum where populations of *S. enterica* were below the limit of detection by 68 days. It is possible that the plastic applied after the cover crop was ploughed in helped to contain the biofumigants in the soil from the sorghum.

Microbial communities

Microbial abundance and composition of soil fungal and bacterial communities were strongly related to soil type. Soil texture was the most determinant variable affecting the microbial community, both by itself and combined with other treatments. Clay loam had more a consistent microbial composition than sandy soil which, according to statistical analysis, was more scattered. Our results are consistent with prior studies (e.g. Johnson et al. 2003, Lauber et al. 2008) that indicated the influence of soil texture on soil microbial composition and activity was as important as soil pH. Physical and chemical characteristics related to soil texture, such as soil moisture and nutrient content, determine the structure of microbial communities. Moreover, nearly identical bacterial communities were found in spatially separated soils with the same soil texture.

The field trial showed that cover crop incorporation and solarisation induced greater changes in bacterial and fungal abundance and composition in sandy soil than in clay soil (Beta diversity analysis). The content, stability, adsorption and binding capacity of aggregates in clay soil increases the resilience of the microbial community to stresses and disturbance (Nannipieri et al. 2003). In our study, there was only a small shift in fungal community abundance in clay loam compared to sandy soil for which the community composition changed more noticeably. Several other studies have also linked fungal communities with soil textural properties. Filamentous fungi grow via expansion of hyphal networks and anything that reduces pore size represents a physical impedance that affects the ability of fungi to penetrate the soil, and hence restricts their capacity to grow and multiply. The large hyphal diameter of basidiomycetes (the second most dominant phylum in our experiment with 12.6% relative abundance) may be a direct impediment to penetration of heavily textured soil (Wakelin et al. 2008). The incorporation of fresh organic matter into sandy soils resulted in a significant alteration of the microbial community that can be potentially used in agricultural systems to improve soil health.

Effect of biofumigant cover crop on microbial communities

Radish as a cover crop affected soil microbial community abundance the most, closely followed by mustard. These two species belong to the Brassicaceae, a family of plants which has been widely reported to produce changes in the soil microbiome, especially plant pathogens. Several studies have shown that chemicals (glucosinolates) released by Brassicas can significantly reduce soil-borne pathogens when incorporated into the soil (Matthiessen and Kirkegaard 2006). Meanwhile, other studies have reported that the suppressive effects of biofumigation on soil pathogens are associated with a general microbial response to the addition of fresh organic matter (Omirou et al. 2011, Wang et al. 2014).

In our experiment, the chemical characteristics of the cover crops may have led to the change in bacterial and fungal communities, and not only as an input of fresh organic matter. This possibility is based on the comparison among brassica and sorghum treatments, all of which added organic matter to the soil; however, microbial communities associated with sorghum were similar to control treatments. Further research is needed to determine the nature of biofumigation activity of brassica crops. Most of the cover crop treatments induced greater changes in the microbial community of sandy soil than clay loam soil. This suggests that biofumigation may yield better results in sandy soil than in clay loam. Again, further testing to determine the nature of response times and rates to biofumigants is needed.

Effect of soil solarisation on microbial communities

Unexpectedly, the effect of solarisation on the soil microbial community was neither significant by

itself nor in combination with cover crops. Many studies that have found that solarisation causes thermal inactivation of microbes and is an effective technique to control pathogens in soil. This method is regarded to quantitatively and qualitatively alter the soil ecosystem and produce a crucial impact on the soil microbiome (Stapleton 2000). It is likely that the timing of the start of the solarisation treatment had the greatest influence on the lack of significant differences. However, it should be noted that the colour of the plastic used may have influenced these results as several studies report that transparent plastic maximises solar heating of soil. Transparent plastic permits the solar energy to move through into the soil, where it is converted into longer wavelength infrared energy, which is trapped beneath the film creating a greenhouse effect. Opaque black plastic does not permit passage of most solar radiation, instead it absorbs incoming solar energy. Only a small portion of the energy is conducted into soil, but most solar energy is lost by re-radiation into the atmosphere (Stapleton 2000).

Evaluation and discussion

Importance of research outcomes

Microbial safety is of paramount importance to the Australian horticultural industry as assurance of food safety is the foremost requirement for the consumer. The Australian vegetable industry's reputation and positive image among consumers is dependent on remaining alert to global trends in food safety, identifying risks as they arise, and proactively developing risk management and monitoring strategies to prevent compromises in produce integrity.

Australian guidelines for fresh produce safety as well as quality assurance programs developed by major retailers, have been produced to assist growers with safe on-farm practices. However, the research on which the guidelines are based are predominantly drawn from international research and may not be applicable to local conditions. This is the first research conducted in Australia on survival of *Salmonella enterica* in soil contaminated with chicken manure and has generated comprehensive data on the survival of *S. enterica* under relevant field conditions, which will inform public health regulatory and enforcement laws mandated through the respective agencies and authorities.

The research provides valuable insight into the agronomic and on-farm characteristics that affect survival of *S. enterica* under Australian conditions. The research has been of particular interest to the team that has published updated 'Guidelines for Fresh Produce Safety 2015' https://freshproducesafety-anz.com/guidelines/ which provides recommendations for exclusion period (days) between application of untreated manure and crop harvest. Previous guidelines have recommended 120 for high risk crops such as leafy greens and herbs; however, this has recently been reduced to 90 days. Our research showed that *S. enterica* survives for at least 100 days under field conditions, particularly in clay-based soils, and we suggest that the guidelines may need further revision.

Use of raw, unaged or uncomposted manure is not recommended in horticultural production, however many growers apply this type of manure to their land without understanding the true risk of microbial contamination to their crops. Through our research we can now provide some understanding of how long *S. enterica* is likely to survive in different soils under different environmental and agronomic practices and when the risk is likely to diminish.

This study also examined potential short-term remediation strategies of cover cropping and/or solarisation to reduce *S. enterica* levels following contamination. Solarisation (black plastic covering the soil) may have potential to promote faster die-off of *S. enterica* providing soil temperatures under the plastic have reached temperatures of 37°C or above for several hours. However, while the use and incorporation of the commercially-available cover crops, Ethopian Mustard, Oilseed Radish and Fumig8tor Sorghum, significantly enriched the soil microbiome after incorporation into the soil they were not effective in promoting die-off of *S. enterica* in soil. This may have been due to the short growing time of the cover crop (1 month) which limited the amount of biomass and consequently biofumigant incorporated into the soil. Further research is required to fully explore the value of cover cropping as a remediation strategy for reclaiming soil contaminated with *S. enterica*. More research is needed to determine the amount of biomass and biofumigant levels required in the soil for die-off of *S. enterica* and how quickly this could occur.

Success of outreach activities

Outreach activities, including research presentations at a number of national and international conferences (see Appendices 7–9), can be used as evidence to confirm that our research has provided important outcomes for industry. Through these presentations, growers and post farm-gate supply chain members, auditors, extension specialists and researchers have recognised the importance of research presented as the first time that an on-farm simulation of a contamination event has been tracked in terms of pathogen survival.

An important aspect of this research has been the successful collaboration between Dr Trevor Suslow and his research group at UC Davis and researchers from the University of Sydney. The collaboration has enhanced the research outcomes through replication of the trials in both countries as well increasing the robustness of results through shared methodologies. Research personnel exchanges funded outside of this project have been extremely valuable with Dr Phan-Thien visiting UC Davis to learn about their research methodologies and Dr McConchie, Dr Phan-Thien and Dr Bell and Dr Suslow presenting their joint research at the Center for Produce Safety Symposium in 2015. Dr Suslow also visited Australia to share his knowledge with growers and researchers.

Our research findings will continue to be reported to a range of audiences including grower groups through presentations at local meetings and the annual AusVeg conference. A number of short, concise fact sheets have been drafted and will shortly be completed to provide the industry with outcomes of our research. Distribution channels for these fact sheets will include FPSC and AusVeg websites.

Capacity building

This project has built skills and capacity in Australian personnel through providing skill training for a number of undergraduate interns and research training opportunities for two postgraduate students: one MAgr (completed) and one PhD who is due to complete in August 2016. The researchers involved in this study, one of which is regarded as an early career researcher, have benefitted enormously through an increase in the breadth and depth of their knowledge and expertise and by expansion of their networks in industry.

Recommendations

This is the first research to be done under Australian conditions on survival of *Salmonella enterica* in soil contaminated with chicken manure and how soil type, temperature, moisture and presence/absence of chicken manure affect its survival. This research also examined short-term remediation strategies of cover cropping and/or solarisation to reduce levels of *S. enterica* in soil following contamination to allow a quick return to safe crop production. From this research, producers need to consider the following farm soil characteristics and agronomic practices when assessing remediation strategies to reduce populations of *S. enterica* in their soil after a contamination event using chicken manure.

1. Farms with sandy soils may have reduced retention of *S. enterica* following a contamination event from use of chicken manure; however this is contingent upon the amount of organic matter provided by the manure or crop residue in the soils which acts as a source of food for *S. enterica*.

2. Farms with clay loam soil can expect *S. enterica* to survive at detectable level in their soils for at least 100 days following a contamination event from use of chicken manure. This may be influenced by the level of organic matter in the soil which would tend to extend survival of *S. enterica*.

3. Soil temperatures above 37°C such as might occur in summer hasten die-off of *S. enterica* in contaminated soil particularly in sandy soil. Clay loam soils provide some protection against high temperatures. Incorporation of manure overrides the suppressive effect of high temperature on *S. enterica* and enhances pathogen survival in both sandy and clay soils.

4. Constant soil moisture promotes survival of *S. enterica* while fluctuating moisture promotes die-off.

5. The use and incorporation of commercially-available cover crops such as Ethopian Mustard, Oilseed Radish and Fumig8tor Sorghum, can enrich the soil microbiome after incorporation but may not be effective in promoting die-off of *S. enterica*. In the study presented here, this may be due to incorporation of small amount of biomass, and consequently biofumigant, in to the soil. Further research is required to fully explore the value of cover cropping as a remediation strategy for reclaiming soil contaminated with *S. enterica*. Research needs to determine the timing and amount of biomass and biofumigant levels required in the soil for effective die-off of *S. enterica*.

6. Solarisation (black plastic covering the soil) may have potential to promote faster die-off of *S. enterica* providing soil temperatures under the plastic have several hours at temperatures of 37°C or above.

7. Untreated or uncomposted manures such as poultry manure can be a source of energy and nutrients for soil borne pathogens. The Guideline for Fresh Produce Management (2015) do not recommend addition of any untreated manure to soils used for production of short term crops such as leafy salad greens or herbs.

8. Unpleasant odour from on-farm use of chicken manure can be minimised through the following:
    - Use composted or pelletised manure for land application which has less odour and human pathogens
    - If aging chicken manure on-farm keep covered and well away from production sites and natural watercourses
    - Incorporate chicken manure into the soil as quickly as possible

- Incorporate chemicals such as biochar, activated carbon, silica gels or zeolite into the manure during composting to reduce odour emissions
- Planting trees and shrubs around the property boundaries can have positive impact on reducing chicken manure odours
- Apply manure in the morning on sunny days when warm air is rising or when the wind is blowing away from neighbours

Scientific refereed publications
None published to date

Intellectual property/commercialisation
No commercial IP generated

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Appendices

Appendix 1
Experimental methods for the laboratory study

Appendix 2
Experimental methods for the field trial

Appendix 3
Draft fact sheet on 'Odour management of chicken manure'

Appendix 4
Draft fact sheet on 'Effects of biofumigants and solarisation on soil microbial communities'

Appendix 5
Poster prepared for the 29th International Horticulture Congress in Brisbane, 17–22 August 2014

Appendix 6
Center for Produce Safety promotional article for joint Australia/US collaborative research on 'Remediation of *Salmonella* infested soils' published in April 2015

Appendix 7
Invited Speaker presentation at the AIFST Microbiology conference in August 2015

Appendix 8
Invited Speaker presentation at the annual FPSC conference in August 2015

Appendix 1

Laboratory Trial Methods

Salmonella Survival Experimental Design

1. 96 Treatments:
    a. 4 inocula: 3 serovars (Sofia, Enteritidis, Montevideo) & 1 cocktail (Enteritidis, Infantis, Montevideo, Typhimurium, Zanzibar)
    b. 3 temperatures (5, 21, 37)
    c. 2 chicken manure (0, 1)
    d. 2 soil moisture (constant, fluctuating) – monitor and adjust by weight
    e. 2 soil type (sandy, clay)
2. 7 sampling times (1, 8, 15, 22, 29, 36, 43 days after inoculation)
3. 3 replicates: 144 samples x 2 lots (2 inocula offset by 2 days)

Experimental Preparation
Preparation of Media

1. Soil extraction media (0.02 M sodium phosphate + 0.1% Tween 20): 44 L each week.

a. Dissolve sodium phosphate and Tween 20 in ultrapure water as follows.

Schott bottle size	$Na_3PO_4 \bullet 12H_2O$(g)	Tween 20 (mL)	Water (mL)
500 mL	3.0	0.4	400
1 L	6.1	0.8	800
2 L	12.2	1.6	1600
	38.0	5.0	5000

b. Autoclave at 121°C for 15 min.
c. Store prepared petri dishes at 5-25°C (i.e. lab room temperature is okay).

2. Phosphate buffer (pH 7.2) stock solution

a. Prepare stock solution by dissolving 34.0 **BBL Phosphate Buffer, pH 7.2** in ultrapure water and make up to 1 L.
b. Prepare working solution by transferring 1.25 mL stock solution to a volumetric flask or measuring cylinder and making up to 1 L with ultrapure water.
c. Dispense 99 mL or 9 mL aliquots into bottles (for convenient diluent).
d. Autoclave at 121°C for 15 min.
e. Store prepared petri dishes at 5-25°C (i.e. lab room temperature is okay).

3. <u>Double strength</u> buffered peptone water: 44 L each week.

a. Suspend **Difco Buffered Peptone Water** dehydrated media in ultrapure water as follows.

Bottle size	Dehydrated media (g)	Water (mL)
500 mL	16.0	400
1 L	32.0	800
2 L	64.0	1600

b. Autoclave at 121°C for 15 min.
c. Store prepared medium at 5-25°C (i.e. lab room temperature is okay).

4. Tetrathionate broth (without iodine): 2.6 L each week. Store in dark.

a. Suspend **Difco Tetrathionate Broth Base** dehydrated media in ultrapure water as follows.

Bottle size	Dehydrated media (g)	Water (mL)
500 mL	18.4	400
1 L	36.8	800
2 L	73.6	1600

b. Do not autoclave. Heat until boiling by one of the following methods. Wear protective gloves to handle the bottles.
    i. Agitate on hot plate with magnetic stirrer.

ii. Microwave (medium power, 2 min) under supervision, ensuring to loosen lid and agitate regularly (e.g. every 15 sec) to ensure broth does not boil over.

c. Allow broth to cool then transfer aliquots of 8.8 mL to sterile 15-mL centrifuge tubes.
d. Store prepared broth base (without iodine solution) in the dark (e.g. covered in foil) at 5-25°C (i.e. lab room temperature is okay). NB TTB must be used immediately once iodine has been added.

5. TTB iodine supplement: 52 mL each week: prepare 500 mL in advance and store in dark. Do not refrigerate.

a. Dissolve iodine crystals and potassium iodide in water as follows.

Iodine (g)	Potassium iodide (g)	Water (mL)
15.0	12.5	50
30.0	25.0	100
150.0	125.0	500

b. Iodine solution is light-sensitive. Store iodine solution in tightly-sealed amber vials or wrapped in foil. 15 mL amber vials are convenient.
c. NB, sterilise aliquots of about 30 mL iodine solution by passing through a sterile 0.22 µm syringe filter into a sterile centrifuge tube.
d. Store iodine solution at room temperature. Do not refrigerate.

6. mBroth: 13 L required twice weekly.

a. Suspend **Bacto M Broth** dehydrated media in ultrapure water as follows.

Bottle size	Dehydrated media (g)	Water (mL)
500 mL	14.5	400
1 L	29.0	800
2 L	58.0	1600

b. Autoclave at 121°C for 15 min.
c. Store prepared medium in the fridge (2-8°C).

7. XLT4: 5 L of plates (about 100 plates per week).

a. Mix **Difco XLT4 Agar Base** and **XLT4 Agar Supplement** in ultrapure water as follows.

Schott bottle size	Dehydrated media (g)	Supplement (mL)	Water (mL)
500 mL	23.6	1.84	400
1 L	47.2	3.68	800

b. Do not autoclave. Heat until boiling by one of the following methods. Wear protective gloves to handle the bottles.
   i. Agitate on hot plate with magnetic stirrer.
   ii. Microwave (medium power, 2 min; high power, 2 min; medium power, 2 min) under supervision, ensuring to loosen lid and agitate regularly (e.g. every 15 sec) to ensure broth does not boil over.
c. Allow to cool to about 60°C (for safe handling) then pour into petri dishes using aseptic technique.
d. Store prepared petri dishes fridge (2-8°C).

8. TSA: 1 L – sufficient for maintenance of cultures and preparation of inoculum.

a. Suspend **Difco Tryptic Soy Agar** or **BBL Trypticase Soy Agar** in ultrapure water as follows.

Bottle size	Dehydrated media (g)	Water (mL)
500 mL	16.0	400
1 L	32.0	800
2 L	64.0	1600

b. Autoclave at 121°C for 15 min.
c. Allow to cool to about 60°C (for safe handling) then pour into petri dishes using aseptic technique.
d. Store prepared petri dishes at 5-25°C (i.e. lab room temperature is okay).

Preparation of Inoculum

1. Streak each serovar (Enteritidis, Infantis, Montevideo, Sofia, Typhimurium, Zanzibar) on to XLD. Incubate overnight (37°C, 18 h).
2. Select a single colony from each plate and sub-culture on to Nutrient Agar. Do 3 times for each serovar. Incubate overnight (37°C, 18 h).
3. Confirm serological identification.
    a. Place one drop of antisera on a glass slide.
    b. Select a single colony from the NA and agitate in the drop of antisera.
    c. Agglutination indicates serological identification.
    d. NB expected serogroups below:

Serovar	**Serogroup**	**Antisera**
Enteritidis	D1	O:9
Infantis	C1	O:7
Montevideo	C1	O:7
Sofia	B	O:4
Typhimurium	B	O:4
Zanzibar	E1	O:3,10

4. Streak cultures of confirmed serovars onto TSA, incubate overnight, then store in containers in fridge for maintenance.
5. Prepare lawn cultures as follows.
    a. Suspend 5 colonies fron NA or TSA in 5 mL of phosphate buffer (pH 7.2) and mix.
    b. Spread 100 µL suspension on TSA and incubate overnight (37°C, 18 h).
    c. Transfer lawn to 100 mL phosphate buffer (pH 7.2) by adding solution to the petri dish, detaching bacteria using a spreader, then pouring it back into the phosphate buffer. NB smaller quantities can be made by directly scraping off colonies with a loop.
6. Estimate the inoculum concentration by measuring the optical density (600 nm) in a spectrophotometer.
    a. In UC Davis, an OD of 0.75 absorbance indicates approx. log 9 CFU/mL. On our old spectrophotometer (teaching lab), an OD of 0.8 indicates approx. log 8 CFU/mL. An OD of 0.85 indicates approx. log 8.8 CFU/mL. I expect OD 0.9 will give log 9 CFU/mL.
    b. The inoculum concentation can be adjusted by dilution, or by centrifuging and resuspending the pellet as necessary.
    c. NB it is easier to produce much higher volumes and concentrations of robust inoculum using a lawn culture than using a broth.
7. There are 504 pots per serovar in this experiment. Therefore approximately 2.6 L of log 6 CFU/mL inoculum is required for each serovar.
    a. Scrape one lawn culture in 100 mL phosphate buffer. Check the OD.
    b. Dilute or add part of a second lawn culture if necessary and adjust to OD 0.8.
    c. Add 50 mL inoculum and make up to 2.6 L with phosphate buffer. Mix well.
    d. For the cocktail inoculum, 10 mL of each of 5 serovars mixed and made up to 2.6 b L.

Inoculation of Soil

1. Add 10 mL inoculum to each pot (log 6-7 CFU).
2. Mix pots on drum mixer for 20 min.
    a. There are 504 pots/serovar and therefore 1008 pots/day.
    b. There are 2 drum mixers with capacity 40 pots each, i.e. 80 pots total capacity.
    c. Therefore there will be 13 pot lots taking a total of 4.5 h.
3. Record the weight of each pot.
    a. Weigh the **odd** numbers with the lids on. Write the weight on the pot.
    b. Weigh the **even** numbers with the lids off. Cover with a gauze square and secure with a rubber band. Write the weight on the pot.
    c. Don't forget the control pots (C1-C8).
4. Further divide the pots into **odd** and **even** numbers.
    a. The **odd** numbers are closed and placed into sealed boxes.
    b. The **even** numbers are placed into boxes covered with gauze secured with tape.

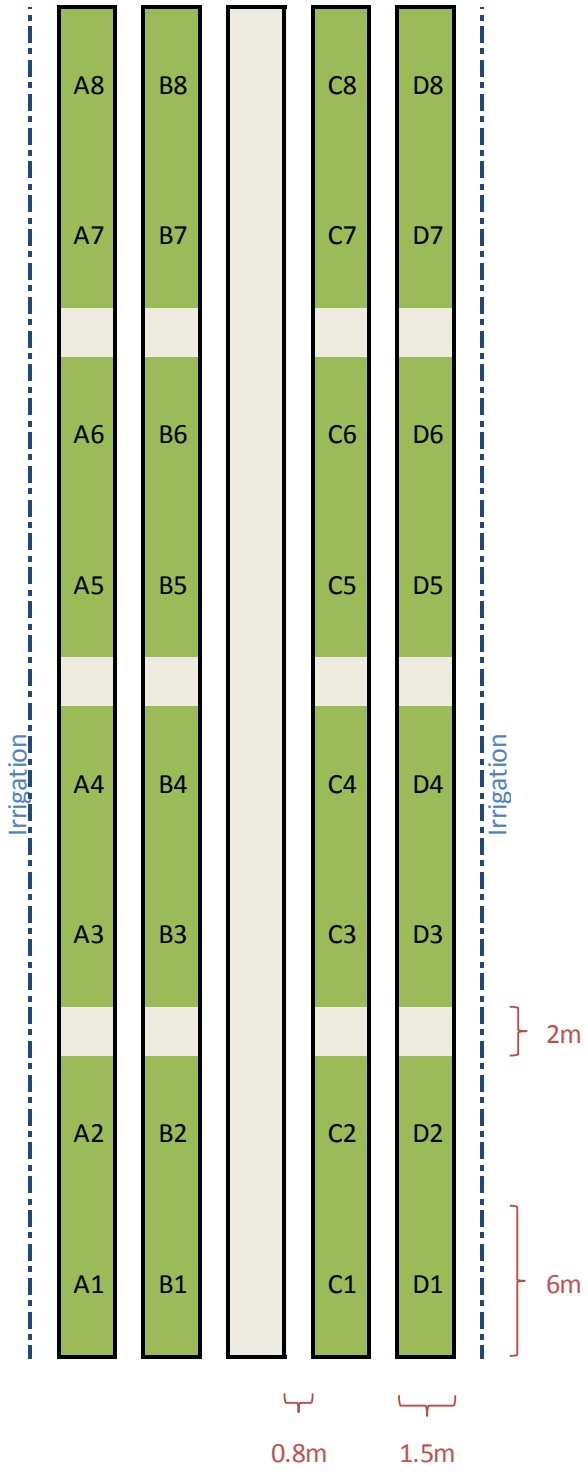
Appendix 2

Field Experimental Design and Protocols

Treatment Details

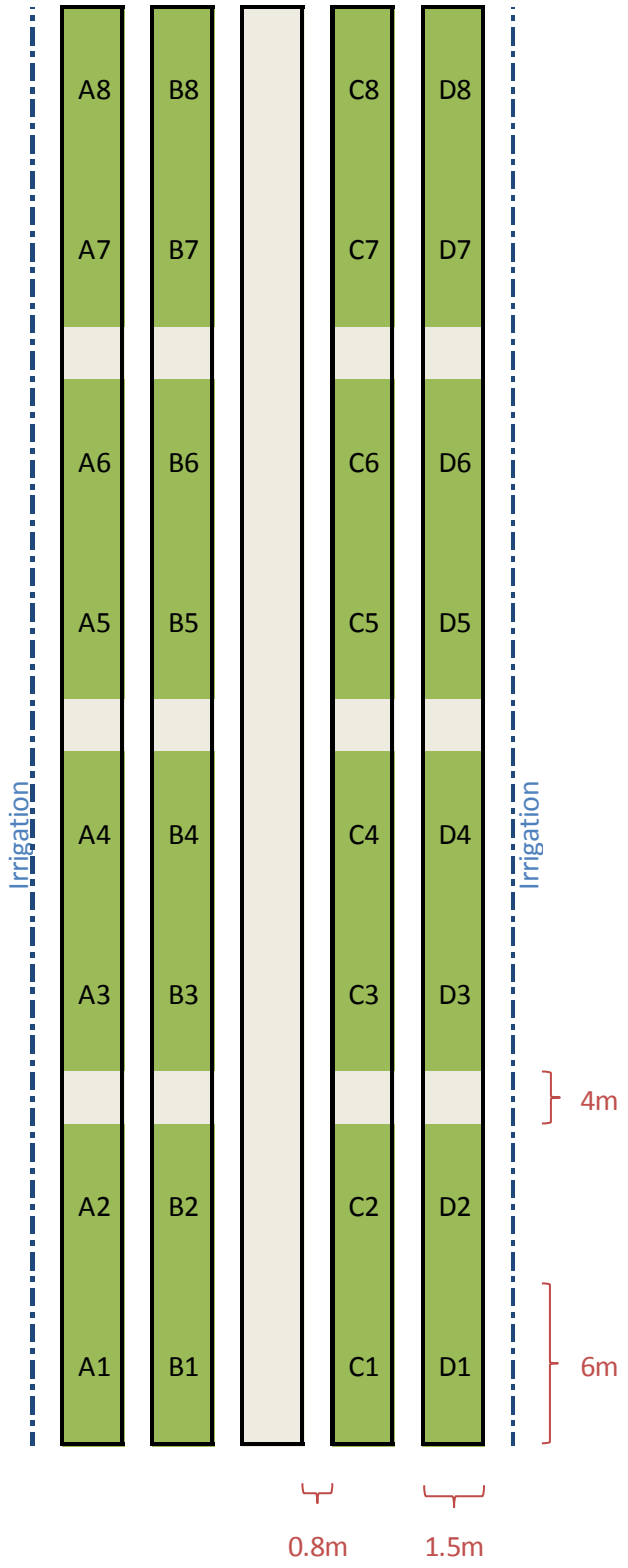
1. Cover crops (4 levels)
    a. No cover crop
    b. Cover crop A: 'Terranova' oilseed radish (*Raphanus sativus*) from Seedforce
    c. Cover crop B: 'Cappuccino' Ethiopian mustard (*Brassica carinata*) from Seedforce
    d. Cover crop C: 'Fumig8tor' sorghum from Pacific Seeds
2. Solarisation: with or without black plastic
3. Soil type: sandy (Karalee) or loamy clay (Pye)
4. Total samples: Quadruplicate plots = 64 samples per sampling time

Field Layout – Karalee Farm, PBI



Plot Coordinates		Treatment	
Block	**Row**	**Wplot**	**Splot**
A	1	Mustard	Solar
A	2	Mustard	No Solar
A	3	Mustard	No Solar
A	4	Mustard	Solar
A	5	No Cover	Solar
A	6	No Cover	No Solar
A	7	No Cover	No Solar
A	8	No Cover	Solar
B	1	No Cover	Solar
B	2	No Cover	No Solar
B	3	No Cover	No Solar
B	4	No Cover	Solar
B	5	Radish	No Solar
B	6	Radish	Solar
B	7	Mustard	No Solar
B	8	Mustard	Solar
C	1	Sorghum	Solar
C	2	Sorghum	No Solar
C	3	Radish	Solar
C	4	Radish	No Solar
C	5	Mustard	No Solar
C	6	Mustard	Solar
C	7	Radish	No Solar
C	8	Radish	Solar
D	1	Radish	No Solar
D	2	Radish	Solar
D	3	Sorghum	Solar
D	4	Sorghum	No Solar
D	5	Sorghum	Solar
D	6	Sorghum	No Solar
D	7	Sorghum	Solar
D	8	Sorghum	No Solar

Field Layout – Pye Farm



Plot Coordinates		Treatment	
Block	**Row**	**Wplot**	**Splot**
A	1	No Cover	No Solar
A	2	No Cover	Solar
A	3	Sorghum	No Solar
A	4	Sorghum	Solar
A	5	Mustard	Solar
A	6	Mustard	No Solar
A	7	Radish	No Solar
A	8	Radish	Solar
B	1	Mustard	Solar
B	2	Mustard	No Solar
B	3	Mustard	Solar
B	4	Mustard	No Solar
B	5	No Cover	No Solar
B	6	No Cover	Solar
B	7	No Cover	Solar
B	8	No Cover	No Solar
C	1	Sorghum	Solar
C	2	Sorghum	No Solar
C	3	No Cover	Solar
C	4	No Cover	No Solar
C	5	Sorghum	Solar
C	6	Sorghum	No Solar
C	7	Mustard	No Solar
C	8	Mustard	Solar
D	1	Radish	No Solar
D	2	Radish	Solar
D	3	Radish	Solar
D	4	Radish	No Solar
D	5	Radish	Solar
D	6	Radish	No Solar
D	7	Sorghum	Solar
D	8	Sorghum	No Solar

Karalee Farm, PBI

Pye Farm

Preparation of Field Sites

1. Basic preparation and weed control.
2. Soil samples collected for baseline characterisation.
3. 5 raised beds formed with 1.5 metre bed width, where the central bed was left fallow as a buffer zone.
4. Plot areas marked out with stakes and spray marker (paint).
    a. PBI: 12 m whole plots and 2 m row breaks
    b. Pye: 12 m whole plots and 4 m row breaks
    c. 0.8 m wheel tracks

Preparation of Inoculum

1. Rifampicin-resistant *Salmonella* Montevideo selected by sub-culturing on TSA with incremental rifampicin amendment. 3.125 mL of rifampicin solution (10 mg/mL) was added to 400 mL media, i.e., final concentration in media was 78 mg/L.
2. Glycerol stock culture was revived on TSA (2 plates). Single colonies from this were streaked onto 20 TSA plates and serotyped. All were positive for serotype B.
3. Lawn cultures of rif-resistant S.Montevideo were prepared on TSARP from the single colony streak cultures after serotyping. Chicken manure pellets were inoculated twice to achieve desired level of contamination.
    a. First inoculation:
        i. On 6 Jan 2015, 5 colonies from a single (serotyped) plate were transferred to 5 mL PBS. This suspension was used to make 20 lawn cultures.
        ii. On 7 Jan 2015, 20 lawn cultures were transferred to 5L PBS. On site, 625mL bacterial suspension was mixed with 2L dechlorinated tap water. Diluted suspension was showered onto 20kg chicken manure pellets while rotating in a conventional brickies cement mixer. The pellets were divided into 2 plastic boxes for drying and storage while loosely covered.
    b. Second inoculation:
        i. On 11 Jan 2015, 15 colonies (from a different single plate) were transferred to 15 mL PBS. This suspension was used to make 100 lawn cultures.
        ii. On 13 Jan 2015, 100 lawn cultures were transferred to 5L PBS. On site 625mL bacterial suspension was mixed with 1L dechlorinated tap water and applied as above.

Lettuce

1. Green oakleaf lettuce planted using a transplanter, with improperly planted seedlings corrected by hand. At Pye Farm, virtually all seedlings were planted manually due to the high clay content.
    a. On 16 Jan, initial planting at PBI and Pye Farms.
    b. On 20 Jan, 90% of seedlings at Pye Farm had died and were replanted.
2. The seedlings were planted in two rows in each bed (excluding the buffer zone) at a spacing of approx. 30 cm.
3. The lettuce was irrigated as required
4. On 6 Feb, the lettuce was incorporated using a rotary hoe.

Weather Monitoring

- Closest weather station to PBI is the BOM weather station at Camden.
- Closest BOM stations to Pye include: Bringelly (Maryland) or Badgerys Creek
- Moisture content measured after each sampling event.
- Temperature logged using Thermochron iButtons at hourly intervals.

Field Treatments

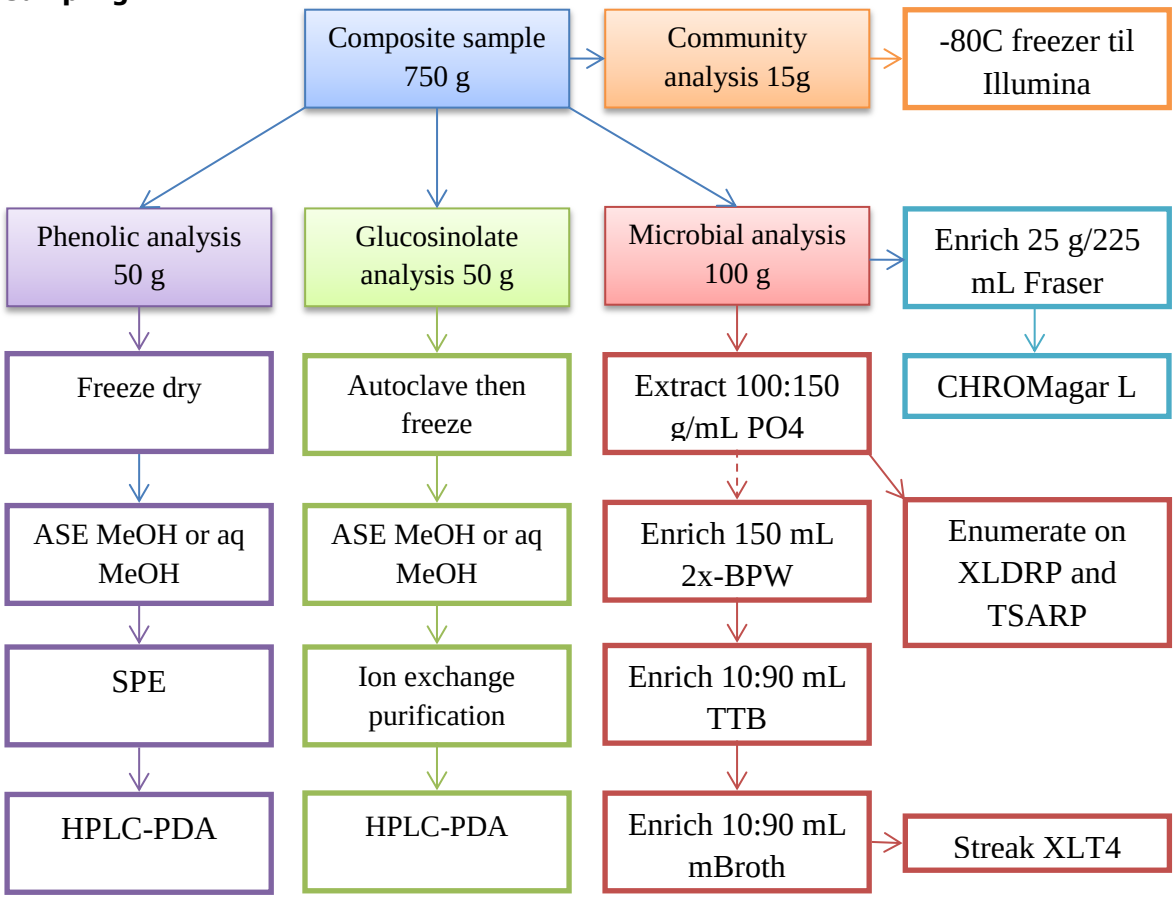
Whole Plot: Cover Crop Application

1. Pre-weigh seeds in paper/plastic bags for convenient manual distribution.
    a. **Sowing rate for radish is 10 kg/ha = 1 g/m^2**. We want a bag of seed to broadcast over 2 m (i.e. 3 m^2), which is easy to estimate by eye. Therefore weigh out **24 × 3 g bags of each seed**. NB, bring some extra – to plant in the buffer zone to estimate biomass at time of incorporation, and in case we drop a bag or something.
    b. **Sowing rate for mustard is 15 kg/ha = 1.5 g/m^2**.

c. Sowing rate for sorghum is 100 plants/m^2. First work out the 100-seed weight and then calculate how much to weigh into each bag. This worked out at **3.1 g/m^2**.

2. Preparation for sowing.
   a. Seed were sown by hand. The radish and mustard were raked in as they needed just 1 cm depth. Sorghum was distributed after making a furrow with rake/shovel.
   b. Black plastic was installed for split plots :
      i. 6 m × 4 splots at each site = 50 m plastic needed on 18 Feb (slight delay).
      ii. 6 m × 12 splots at each site = 150 m plastic needed on 23 Mar.
   c. We used 6 pegs per 6 m splot (3 each side) be adequate?
   d. Holes cut with Stanley knife for sampling, then taped with cloth tape after sampling.

Sampling



Salmonella Analysis

- Approx. 100 g composite soil weighed.
- 150 mL PO4 buffer added – gravimetric diluter feature to automatically adjust sample/solvent ratio **not** used.
- After stomacher-mixing, extracts allow to settle for 15 min. Then 1 mL aliquot transferred to microtube for spiral-plating.
- Extracts enumerated on:
  - XLD – amended with (XX mg/mL or uM) rifampicin and (XX mg/mL or uM) sodium pyruvate.
  - TSA – amended with (XX mg/mL or uM) rifampicin and (XX mg/mL or uM) sodium pyruvate.
  - TSA – amended with (XX mg/mL or uM) rifampicin and (XX mg/mL or uM) sodium pyruvate and (XX mg/mL or uM) **pentachloronitrobenzene**.
  - TSA – amended with (XX mg/mL or uM) rifampicin and (XX mg/mL or uM) sodium pyruvate and (XX mg/mL or uM) **cycloheximide**.

Odour Management of Chicken Manure at Land Application Sites

The land application of chicken manure is widely practiced in Australia and all over the world, and considerable amount of chicken manure has been used to produce vegetable crops such as lettuce, eggplant, cabbage, broccoli peas, tomatoes, brassicas and cauliflower.

Pros

Rich in plant nutrients
Cheap alternative
Release nutrients quickly
Improve soil properties
Benefits for soil biota

Cons

Nutrient leaching into ground water
Loosing nutrient in gaseous form
Changing chemical properties of soil
Health problems (*Salmonella*)
Unpleasant odours & dust

Unpleasant Odour of Chicken Manure

Odours originating from chicken manure are a result of a combination of 60-150 odour-producing compounds including volatile fatty acids, mercaptans, esters, carbonyls, aldehydes, alcohols, ammonia, and amines. These odours are mainly generated by the anaerobic decomposition of faecal materials, feathers, dust and bedding materials. The breakdown of sulphide containing compounds produces hydrogen sulphide, mercaptans and organic sulphide, while the breakdown of nitrogen containing compounds releases ammonia, amines, indole and skatole. All of these compounds collectively contribute for unpleasant odours. These odours have negative impact on the welfare of birds as well as environmental, health and socioeconomic issues in the society.

How to Control the Odour of Chicken Manure?

Odour sources of chicken manure can be classified into two broad categories: buildings and facilities and land application sites. The odour control measures can be applied at the chicken sheds/ building and facilities, manure storage and at the land application site and for an effective odour control, it is necessary to take suitable measures at all levels. Proper measures to control odours of chicken manure at first stage (at building and facilities) contribute to reduce odour levels and the potential for conflicts at the storage area and land application sites. Designing of chicken sheds in a suitable site with necessary facilities such as proper temperature control and ventilation systems, ensuring birds' health and adherence to proper management practices are essential in reducing odour emissions at chicken sheds and buildings.

Mostly, chicken manure has to be stored before the land application either within the facilities or in the land application site due to various reasons such as limited cleanout time, availability of resources and equipment and weather conditions. In order to reduce odour emission during storage, it is essential to provide a good protection for manure storage piles from unfavorable weather conditions. Aerobic and anaerobic processing under proper

supervision and composting of chicken manure can also minimize the unpleasant odour emissions during storage.

Odour Control at Land Application Sites

The most significant complaint about poultry manure odour by the public is during and after agricultural land applications. Many factors including local regulations, type of storage that manure has been stored, method of transportation, weather conditions such as rain and wind direction, time period, number of birds contributed for manure, distance to neighbours and public areas and distance to property line can influence the levels of odours that are acceptable to public in terms of frequency, intensity, duration and offensiveness at land application sites. In order to minimize odour issues, it is necessary to consider all these factors during land application and adopting following measures may help to minimize the potential conflicts related to odour issues.

- Biological and chemical treatments during storage of chicken manure can have a significant effect on reducing odour issues at land application sites.
- Application of dried chicken manure is less problematic as odour arising from dried chicken manure is less offensive compared to the fresh manure.
- Transportation of dried chicken manure to the land application site is also less problematic compared to the fresh manure. It is also essential to avoid spillage on public roads during transportation.
- Surface spreading of chicken manure on top of the soil without incorporation into the soil can cause more unpleasant odours. Hence, incorporation of chicken manure into the soil immediately after land application or injection is essential. Broadcasting, plowing and harrowing can cause more serious odour issues.
- Incorporation of chicken manure into the soil as quickly as possible can have a positive impact on reducing odour issues.
- Incorporation of chemicals into the soil that having potentials in reducing odour emissions from chicken manure may help to reduce the odour emission after land applications.
- Developing good communication channels and relationships with neighbours regarding spreading times and dates.
- Taking pro-active approaches are essential in managing odour issues by engaging all other stakeholders such as end-users and regulators as well. Farmers must develop acceptable odour management practices and establishing procedures for verifying and must respond to odour complaints.
- Application of manure in morning on sunny days when air is warming and rising instead of afternoon, choosing suitable days such as when the wind is blowing away from neighbors (both wind direction and intensity) and choosing weekdays as neighbours have a higher probability of being away from home can also help to minimize the odour complaints.
- Planting trees and shrubs around the property boundaries can have positive impact on reducing chicken manure odours.

Salmonella Factsheet 3

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EFFECTS OF BIOFUMIGANTS AND SOLARISATION ON SOIL MICROBIAL COMMUNITIES

Biofumigation is a technique used for natural control of pests and diseases in soil. This method relies on chemicals, such as *glucosinolates* and *isothiocyanates*, that are released from plants such as brassicas. Soil biofumigation has been increasingly adopted worldwide and specialised varieties of mustard, sorghum and arugula with elevated levels of glucosinolates have been developed. Biofumigation offers an economic and sustainable alternative to synthetic compounds and have become particularly important due to reduction in use of the widely used chemical fumigant, methyl bromide[1].

Soil solarisation is a non-chemical method used for controlling soil-borne pests and diseases. Soil is heated by the sun after covering with black or clear plastic sheeting for 4–6 weeks. In summer, the soil surface may reach up to 60 °C during the hottest part of the day and soil temperatures can increase up to 30 °C at depths of 10–15 cm. This method can kill some soil organisms within days but it is recommended that the plastic remain in place for longer periods to control other pests and diseases. Heat-tolerant fungi and bacteria cannot be controlled effectively with soil solarisation.

Both techniques have been used in studies investigating alternative methods for control of soil pathogens. However, the direct influence of biofumigants on the non-targeted component of soil microbial communities is poorly understood. Similarly, little information is available on the interactions among each method and pathogenic and non-pathogenic microorganisms [2,3].

Next generation DNA sequencing technologies offers new options to characterise the taxonomic, phylogenetic, and functional diversity of soil microbial communities at an

Soil contains a total of 4–5 ×10^{30} microbial cells[4]

The functional role of microbes in soil include [3]:

- Nutrient cycling
- Biological regulation
- Decomposition of organic matter
- Formation of humic compounds and soil structure
- Degradation of pollution

unprecedented level of detail, as well as identify many individual species in individual soils and across broad spatial gradients. DNA sequencing is a high-throughput, streamlined, scalable, highly accuracy and cost-effective methodology.

Case study

We aimed to analyse the effects of three biofumigant cover crops (mustard (*Brassica carinata*), radish (*Raphanus sativus*) and sorghum (*Sorghum* sp., Fumig8tor™)) alone and in combination with a solarisation treatment, on the soil microbial community in sandy and clay loam soil. The populations of microbes in the soil were characterised using DNA sequencing technology.

Results

Overall, greater changes were found in abundance of bacteria compared to fungi. Changes in bacterial and fungal diversity were significantly influenced by soil texture such that higher microbial variation was detected in sandy soil compared to clay loam soil. The two brassicas, radish and mustard, significantly altered soil microbial abundance, while sorghum and solarisation had no significant influence on microbial abundance. Brassicas induced greater changes in soil bacterial and fungal communities, mainly with an increase in non-pathogenic microbes.

Conclusion

This evidence supports the claim that pathogens are suppressed by biofumigant cover crops. Future research should aim to analyse the effect of biofumigation at different times after incorporation and to rigorously test the effect of solarisation.

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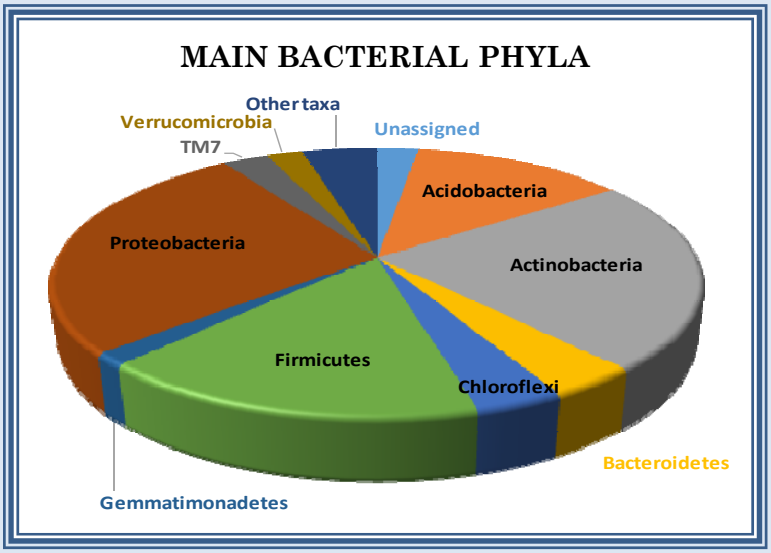
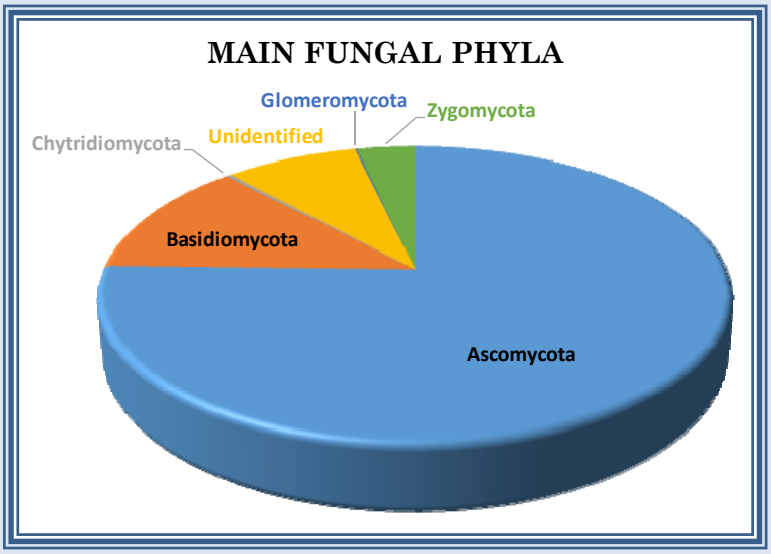
[1] Matthiessen & Kirkegaard (2006) *Critical Reviews in Plant Sciences* 25, 235–265
[2] Larkin *et al.* (2010) *Plant Disease* 94, 1491–1502
[3] Omirou *et al.* (2011) *Microbial Ecology* 61, 201–213
[4] Rastogi & Sani (2011) Microbes and Microbial Technology, 29–57, Springer





Analysis of similarity (ANOSIM) comparing bacterial and fungal community composition between biofumigant cover crops in clay loam soil and sandy soil. An R-value closer to 1 indicates a significant effect.

Soil type	Treatment	Bacteria	Fungi
Clay soil	Mustard	0.17	0.07
	Radish	0.24	0.10
	Sorghum	0.06	0.04
	Control	0.01	0.03
Sandy soil	Mustard	0.19	0.17
	Radish	0.21	0.19
	Sorghum	0.04	0.02
	Control	0.03	0.05

This research was done by **Luz Stella Gonzalez Rubio** as part of the requirements for a Master of Philosophy at the University of Sydney in the Faculty of Agriculture and Environment

November 2015

For more information about project design and other research see *Factsheets 1 and 2*

For more information contact: **Prof Robyn McConchie** (email: robyn.mcconchie@sydney.edu.au)

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

Project Details

PROJECT VG13039

CHIEF INVESTIGATORS

A/Prof Robyn McConchie
Faculty of Agriculture and Environment, The University of Sydney, NSW, Australia

Prof. Trevor Suslow
Center For Produce Safety, The University of California, Davis, CA, USA

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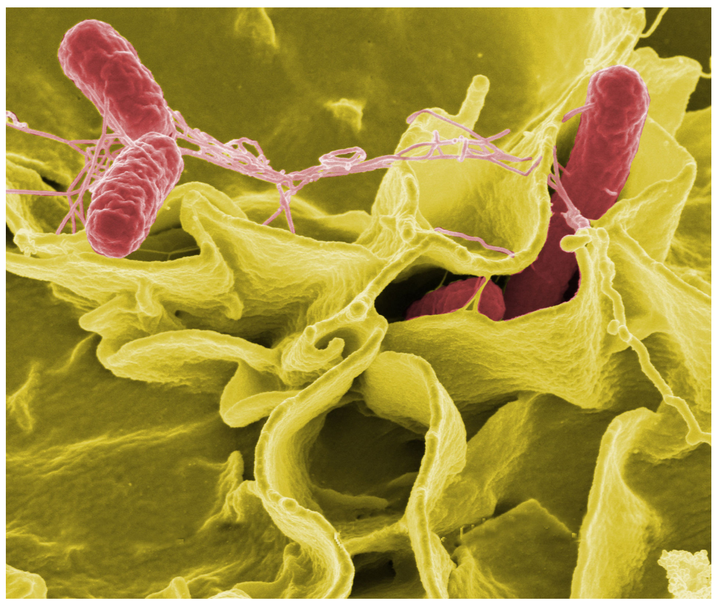
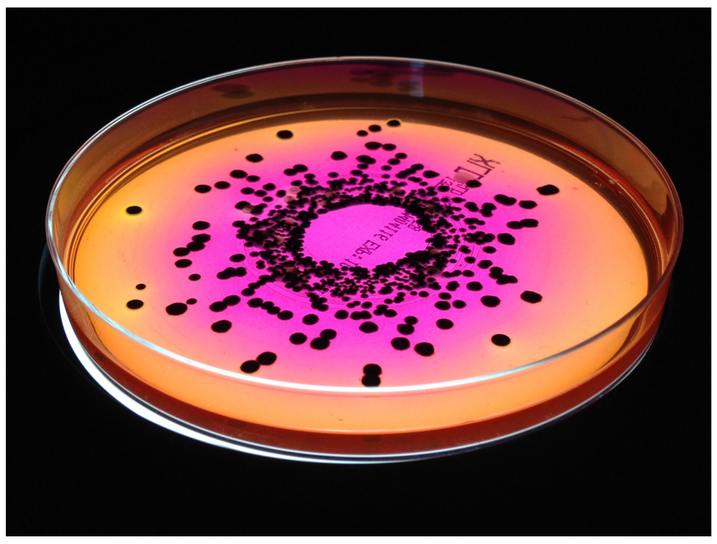
Horticulture Australia





SALMONELLA

- Optimal growth temperature of Salmonella: 35 to 43^{0}C
- Most serovars can grow as low as 7^{0}C
- In general, between 105-106 cells are needed to be consumed to cause illness (Lawley et al. 2008)
- Over 99% of human Salmonella spp. infections are caused by S. enterica subsp. enterica (Crum-Cianflone 2008).

Field Trials

FIELD TRIAL 1

Characterise Salmonella persistence in soil amended with chicken manure under various cover crop regimes: selected mustard, buckwheat and sorghum varieties with biofumigant/ antimicrobial traits.

NB Brassica spp. are known to contain glucosinolates that hydrolyse in the presence of myrosinase to generate biofumigant isothiocyanates.

NB Phenolic compounds in certain buckwheat and sorghum varieties are suggested to have some antimicrobial activity that may affect populations of pathogenic bacteria.

FIELD TRIAL 2

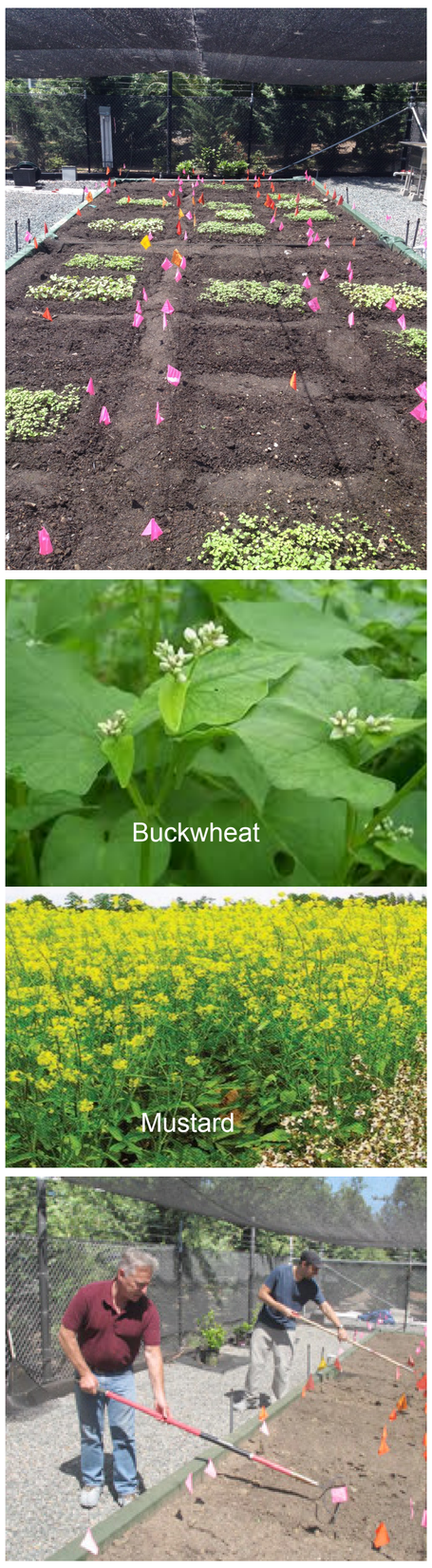
Biofumigant cover crop rotations will be trialed in combination with solarisation to enhance die-off of Salmonella.

FIELD TRIAL 3

The biofumigant cover crop treatments will also be tested in combination with interval flooding as previous research in the USA has indicated this may also reduce pathogen populations.

Laboratory Studies

Characterise the growth and survival of Salmonella under varying conditions: different serovars, soil types, temperatures, moisture profile, and chicken manure.



Introduction

Fresh produce is an important part of a healthy diet. However, bacteria from soil may be conveyed to produce by direct transfer or water splash, and poses a food safety risk in produce that is typically consumed raw or has minimal processing. This is a major challenge for the fresh food produce industry.

Salmonella is the second highest cause of food-borne disease in Australia. Improper use of animal manures during vegetable production is potentially a significant source of pre-harvest contamination. In the US, microbial contamination of soil has led to loss of prime growing locations due to the lack of mitigation strategies to eliminate persistent contamination.

How does contamination occur?

Fresh produce can be contaminated at any point in the production chain:

- irrigation water
- wild or domestic animals
- human handling
- harvesting equipment
- transport containers
- wash water
- transport vehicles
- processing equipment
- inadequately composted manure

Chicken manure is rich in N-P-K and is a great soil amendment, but fresh or improperly composted manure is implicated in preharvest contamination of vegetables

In Australia most growers use composted organic amendments that are certified, while most salad producers do not use manure amendments at all because of the risk

However some growers continue to use aged or stockpiled manure and/or litter, not necessarily composted, which poses a risk of soil contamination

There is little research under Australian conditions on survival of Salmonella and Listeria in vegetable farms using chicken manure.

Project Aims

This project seeks to develop and validate strategies to reduce or eliminate the presence of naturally-occurring human pathogens in soil and thereby improve food safety at the farmgate.

Laboratory and field trials to optimise the use of low-residue 'bio-fumigant' cover crops (e.g. Brassica spp.), which naturally release antimicrobial chemicals upon degradation, as a stand-alone strategy and in combination with solarisation or interval-flooding treatments.

Monitor environmental populations of Listeria spp. and the broader microbial community to explore the potential usefulness of treatments to control other human pathogens.

Analysis of national climate data to validate the extrapolation of solarisation results.

Desk-top study of odour pollution associated with chicken manure amendments and potential control measures.

Outcomes

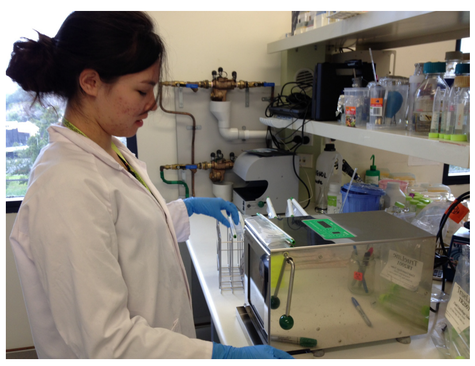
The anticipated outcome of this project is a set of options that growers may employ to effectively manage soil that has been contaminated with Salmonella, and potentially other pathogens, as a critical dimension to minimise microbial food safety risks in produce. The study will also provide for the first time, an important baseline study of survival of the key Australian Salmonella serovars under different soil, temperature and moisture conditions.

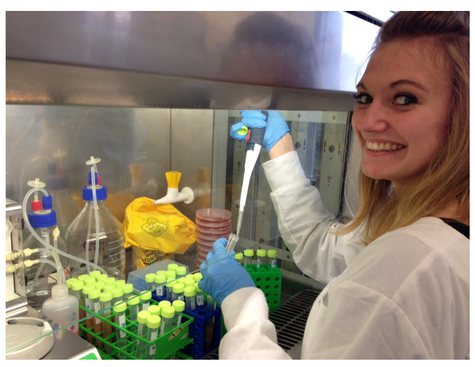
FACT SHEETS FOR GROWERS

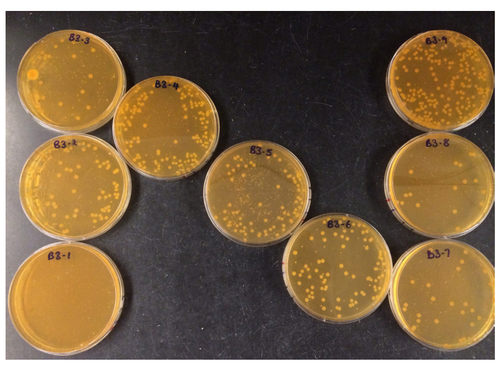
- Persistence of Australian Salmonella serovars in amended and non amended soils
- Potential for risk Listeria sp and L. monocytogenes in amended and non amended soils
- Efficacy of cover crops and solarisation to expedite die-off of Salmonella in contaminated soils
- Best practice re-plant of vegetables in previously contaminated soils
- Best practice guidelines for safe use of chicken manure amendments.

CONTACTS

Robyn McConchie: Robyn.McConchie@sydney.edu.au
Kim-Yen Phan-Thien: Kim-Yen.Phan-Thien@sydney.edu.au

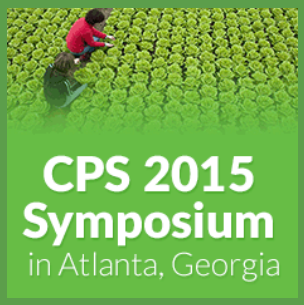






IHC 2014 Brisbane
17-22 August 2014

29th International Horticultural Congress 2014, Brisbane, Australia



A CLOSER LOOK

Research

CPS Campaign for Research Dollars at Work
April 2015

REGISTER HERE - 2015 PRODUCE RESEARCH SYMPOSIUM

A new monthly series reporting to the produce industry on CPS research projects.

Treatments may speed vegetable replanting of *Salmonella*-contaminated soil

Simultaneous field trials are being conducted half way around the world to determine whether cover crops, soil or bed solarization, or a combination of both can help remediate*Salmonellaenterica*-contaminated soil.

The research is being led by Trevor Suslow, University of California Extension Research Specialist in the Department of Plant Sciences, Davis, California; along with co-investigator Robyn McConchie, Associate Professor in the Department of Plant and Food Science and department head, University of Sydney, Australia.

International collaborations of this type benefit both countries as well as speed the research since two sets of trials are conducted in tandem, McConchie said. "We can come to recommendations a lot faster by leveraging off of each other and sharing information," she said of the research, now in its second year." Also, Trevor has had a long experience in fresh produce safety research, and we're benefiting from sharing his information to fast-track our research in Australia as well."

Dr. Trevor Suslow
University of California, Davis

Suslow said he sees advantages when projects -- such as this one, which arose directly from a natural on-farm contamination incident -- present opportunities to work together internationally.

"Certainly in my mind, these are among global issues that the produce industry faces. And to the extent that you can have trials that complement each other on more of an international scale, it helps support the overall usefulness of the data coming out," Suslow said.

Ultimately, McConchie said she hopes their work will yield data on which they can make science-based recommendations for growers with *Salmonella* contamination in their fields. "If you detect persistent*Salmonella* in your soils or on your crop, you have to plow (your crop) under, and that's very costly," she said." There is a lot of uncertainty about how long to withhold replanting without the risk of contamination of that new crop as well. "From a scientific point of view, it's important to also know what's happening to the microbial diversity of

the soil so we can get a handle on what's responsible for any die-off."

McConchie stated they also plan to publish the results in a peer-reviewed journal so quality assurance agencies can potentially develop guidelines for using cover crops to ameliorate *Salmonella*-contaminated soils. Currently, a dearth of practical scientific data exists on survival of *Salmonella* on vegetable farms using chicken manure.

Dr. Robyn McConchie
University of Sydney

Both McConchie and Suslow are following the same research protocol and conducting the same analyses. The main difference is in cover crop varieties. McConchie is using Fumig8tor sorghum, an Egyptian mustard and a radish, all commonly used by Australian growers for their soil pathogen- or nematode-suppression biofumigation traits. Suslow is using two mustards and a buckwheat, all of which are used by Northern California growers.

"These fit into something that growers are familiar with, that growers are doing already,"Suslow said. "They're relatively low cost and our studies are focused on a quick turn-around rather than the typical growth phase for cover crops."

The trials involve 5-by-5-meter plots treated with a chicken manure-chicken litter amendment. A crop of baby lettuce or spinach is then planted, and the "contaminated" crop plowed under. Then the treatments are applied. They involve individual cover crops, solarization alone or solarization combined with a cover crop. The solarization treatments also involve two different moisture regimes -- fluctuating and continuous -- and two different durations -- 30 days or 45 days. One plot will be left untreated as the control.

During the treatments, Suslow and McConchie will collect several sets of soil samples to measure levels of glucosinolates and phenolics -- natural plant compounds produced by the cover crops that have antimicrobial activity. They also are using next-generation sequencing technologies to assess changes in the soil microbial communities. The final step involves disking under the cover crops or removing the solarization plastic, replanting with baby lettuce and spinach, and testing for contamination at typical commercial maturity.

Although the project focuses on *Salmonella*, the researchers also are measuring *Listeria monocytogenes* populations to determine what effects the crop residue treatments have on build-up of this potential soil-borne foodborne pathogen.

McConchie will present their findings at the Center for Produce Safety Symposium, June 23-24, in Atlanta.

View the research abstract proposal: "Remediation and recovery measures to expedite planting or replanting of vegetables following soil contamination by *Salmonella enterica*".

About CPS
The Center for Produce Safety (CPS) is focused exclusively on providing the produce industry and government with open access to the actionable information needed to continually enhance the safety of produce. Established by public and private partnership at the University of California, Davis, initial funding for CPS was provided by the California Department of Food and Agriculture, the University of California, Produce Marketing Association and Taylor Farms. Ongoing administrative costs are covered by the Produce Marketing Association, enabling industry and public funds to go exclusively to research.

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Fresh Produce Safety

Robyn McConchie
Faculty of Agriculture and Environment
The University of Sydney

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About the FPSC

- Established as Not-For-Profit in May 2014 after 2 years of industry consultation
- Affiliated with the Center for Produce Safety in the US
- Website:

http:/freshproducesafety-anz.com/

- Monthly Newsletters – 500 subscribers
- Fortnightly Updates
- Annual Conference

FRESH PRODUCE SAFETY

AUSTRALIA & NEW ZEALAND

FPSC Objectives

Research

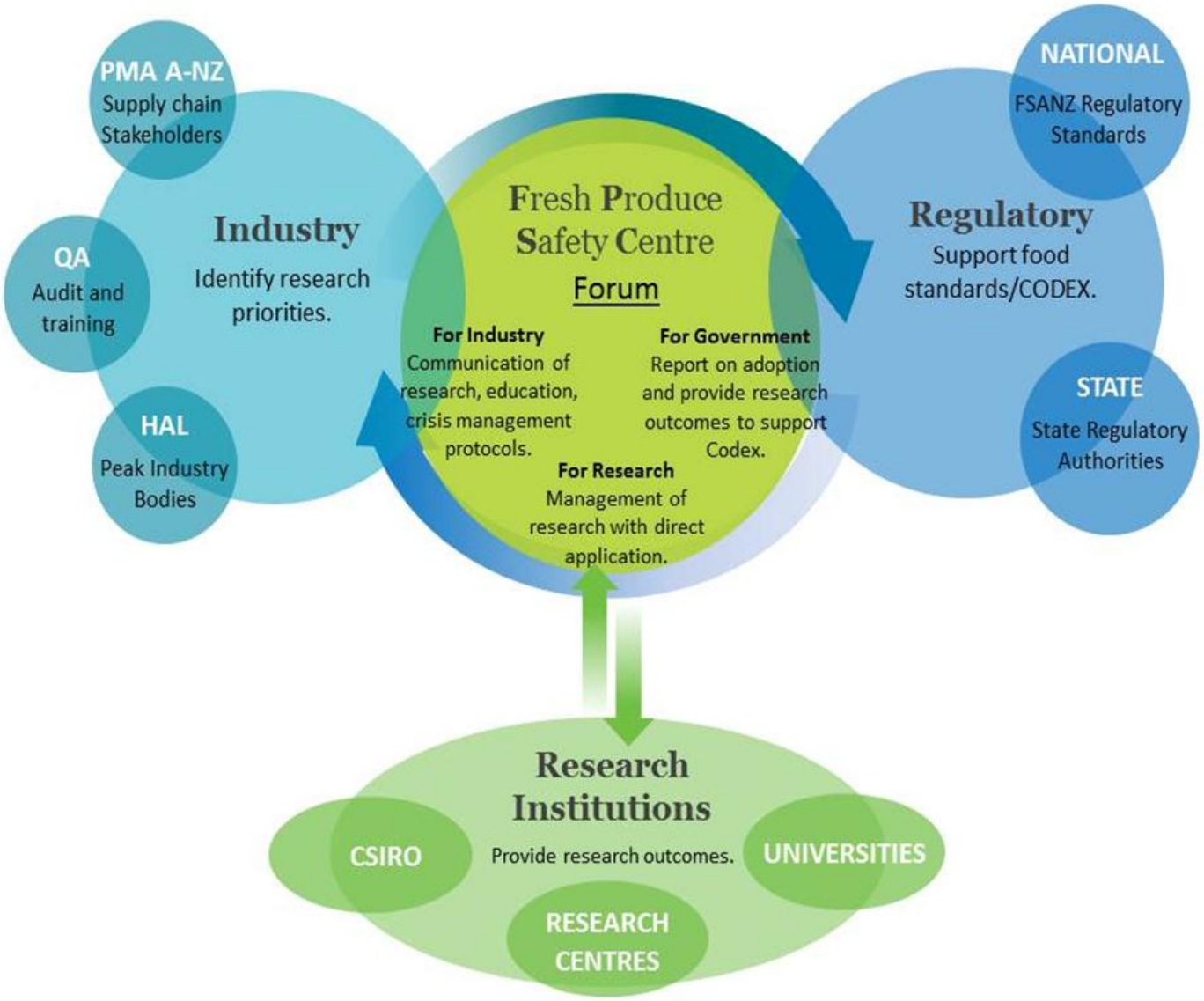
- Identify priorities for research into food safety
- Commission & manage research projects

Outreach

- Increase awareness, provide information, news, education
- Forums and conferences

Consultation

- Across all sectors of fresh produce supply chain
- Regulatory authorities
- International organisations (e.g. Center for Produce Safety)
- Crisis management coordination


PMA A-NZ
Supply chain Stakeholders
QA
Audit and training
HAL
Peak Industry Bodies
Industry
Identify research priorities.
Fresh Produce Safety Centre
Forum
For Industry
Communication of research, education, crisis management protocols.
For Government
Report on adoption and provide research outcomes to support Codex.
For Research
Management of research with direct application.
NATIONAL
FSANZ Regulatory Standards
Regulatory
Support food standards/CODEX.
STATE
State Regulatory Authorities
Research Institutions
Provide research outcomes.
CSIRO
UNIVERSITIES
RESEARCH CENTRES

Led by Industry

- Trans-Tasman
- Multi-sector, multi-discipline

Supported by Industry

Research Projects 2014/15

1. Understanding the Gaps – A Food Safety Literature Review

- Focus is on microbiological contamination
  - Agricultural (pre-harvest) water
  - Organic inputs and composting
  - The storage environment
  - Other production inputs
  - Plus interaction of sanitisers with fungicides
- The latest in best practice and identifying gaps in our knowledge for future R&D
- Insights that can be incorporated into the Guidelines

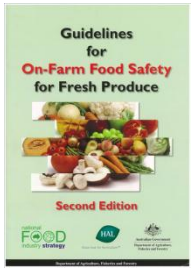
Full Research Partners

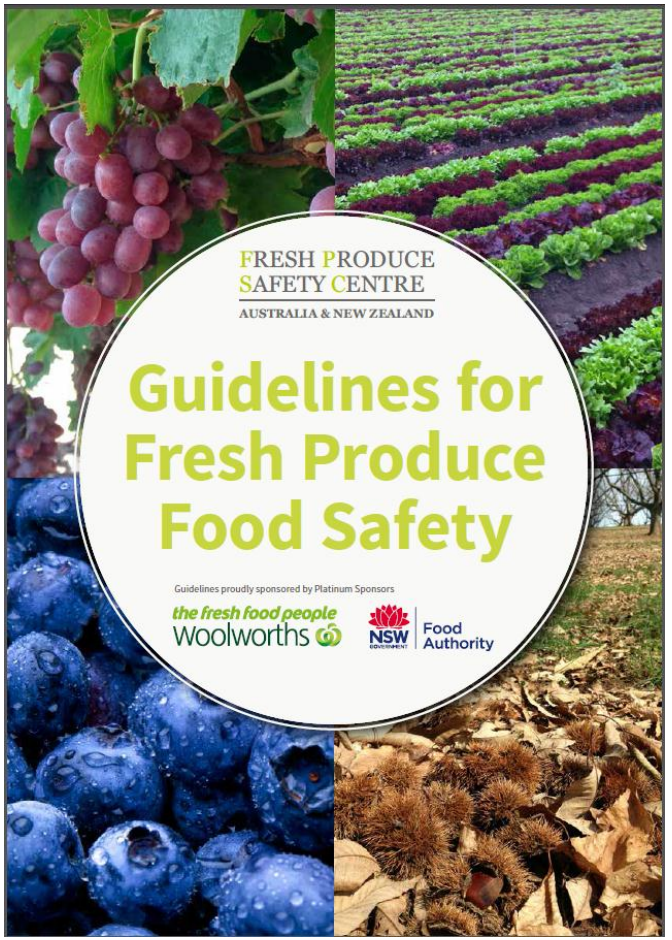
Associate Research Partners

Research Projects 2014/15

2. Review and update the Guidelines for On-Farm Food Safety for Fresh Produce

- Guidelines for Fresh Produce Food Safety
- For whole of supply chain
- Australia and New Zealand
- Additional content including traceability, crisis management, regulations
- Launched 11th Aug 2015
- Sponsored by Woolworths, NSW Food Authority, Freshcare, AusQual, N2N, Fresh Select





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Research Projects 2014/15

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

- Collaborative project with the US Center for Produce Safety and Australian fruit and vegetable industries
- Topics based on priorities for both countries
- University of Sydney collaboration with UC Davis
- Jointly funded by CPS and HIA

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

Robyn McConchie[1], Trevor Suslow[2], Kim-Yen Phan-Thien[1], Tina Bell[1], Mulatua Hailu Metaferia[1], Adrian Sbodio[2] & Tatjana Matic[1]

[1]The University of Sydney

[2]University of California, Davis

Introduction

- Fresh produce is important part of healthy diet
- Produce eaten raw e.g. leafy greens and fruit are vehicles for transmission of human pathogens
- Bacterial pathogens major contributors
- Salmonella is widespread, many serovars, and the most commonly reported pathogen in Australia
- In Australia *Salmonella* was the second highest cause of notified cases of food-borne illness (Chinivasagam *et al*., 2012)
- Contaminated at any point in the production chain: irrigation water, inadequately composted manure, wild or domestic animals, human handling, harvesting equipment, transport containers, wash water and processing equipment

Introduction

- Soil amendments with chicken manure (N-P-K, physical properties), but associated with *Salmonella* (Runge *et al*., 2007)
- Pre-harvest contamination of vegetables is mainly from the use of fresh/improperly composted manure (Wilkinson, 2011)
- Australia - most salad producers do not use manure amendments because of the risk
- In US use of heat treated chicken manure pellets (standards)

Project Aims

- **Pot Study (Australia)**
  - *Salmonella* recovery under controlled conditions
  - Soil type, manure, temperature

Mesocosm Trial (US)

  - *Salmonella* recovery under field conditions at a small scale
  - Cover crop

- **Field Trials (Australia and US)**
  - *Salmonella* recovery under field conditions
  - Soil type, cover crop, solarisation
  - Presence/absence *L.monocytogenes*

Pot Study:
Soil Type, Temperature, ± Manure

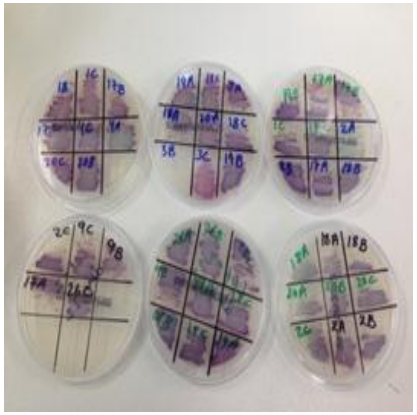
Inoculate with *Salmonella*

Incubate

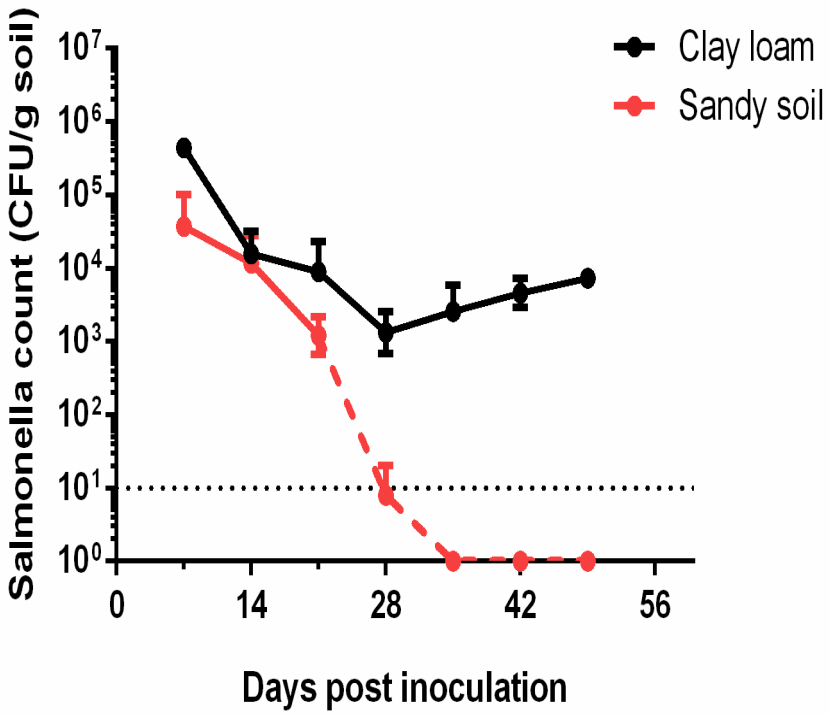
Extract

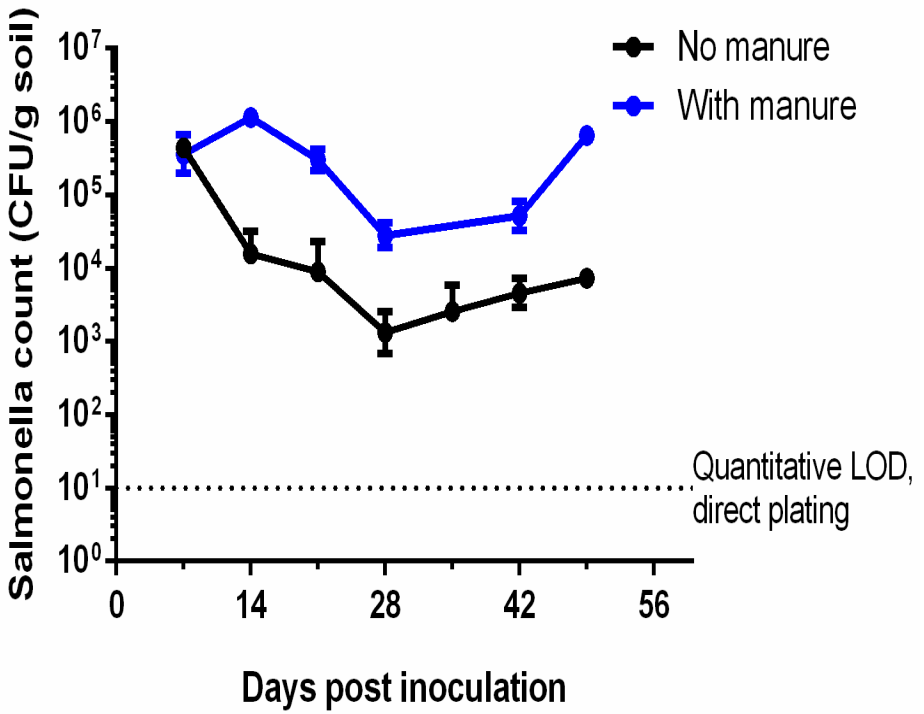
Enumerate

Enrichment



Pot Study: *Salmonella* Cocktail Recovery over Time

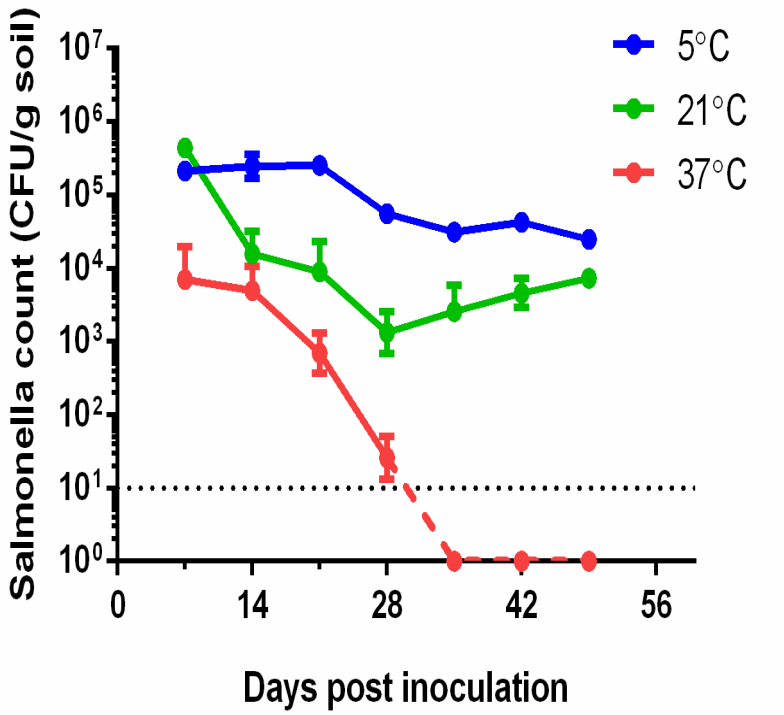




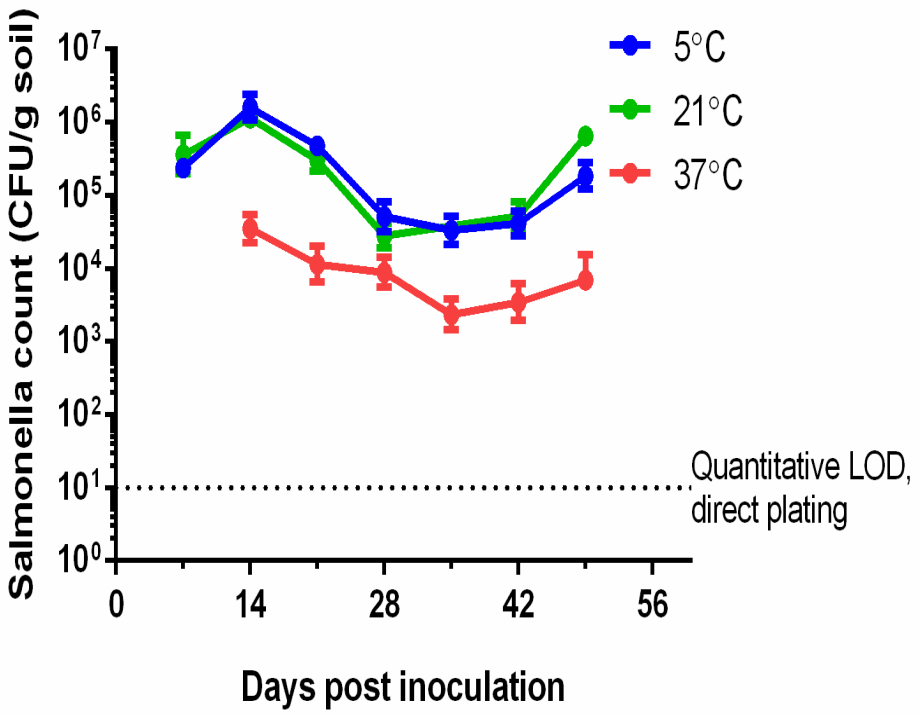
Clay loam promotes survival of *Salmonella* compared to sandy soil

Presence of manure promotes *Salmonella* survival

Pot Study: *Salmonella* Cocktail Recovery-Temperature



High temperature (37°C) reduces *Salmonella* survival in 4 weeks



Effect of high temperature negated by addition of manure

Field Trial: Contaminate – Lettuce Crop – Cover Crop ± Solarisation

Inoculate Manure

Manure Application

Plant Lettuce

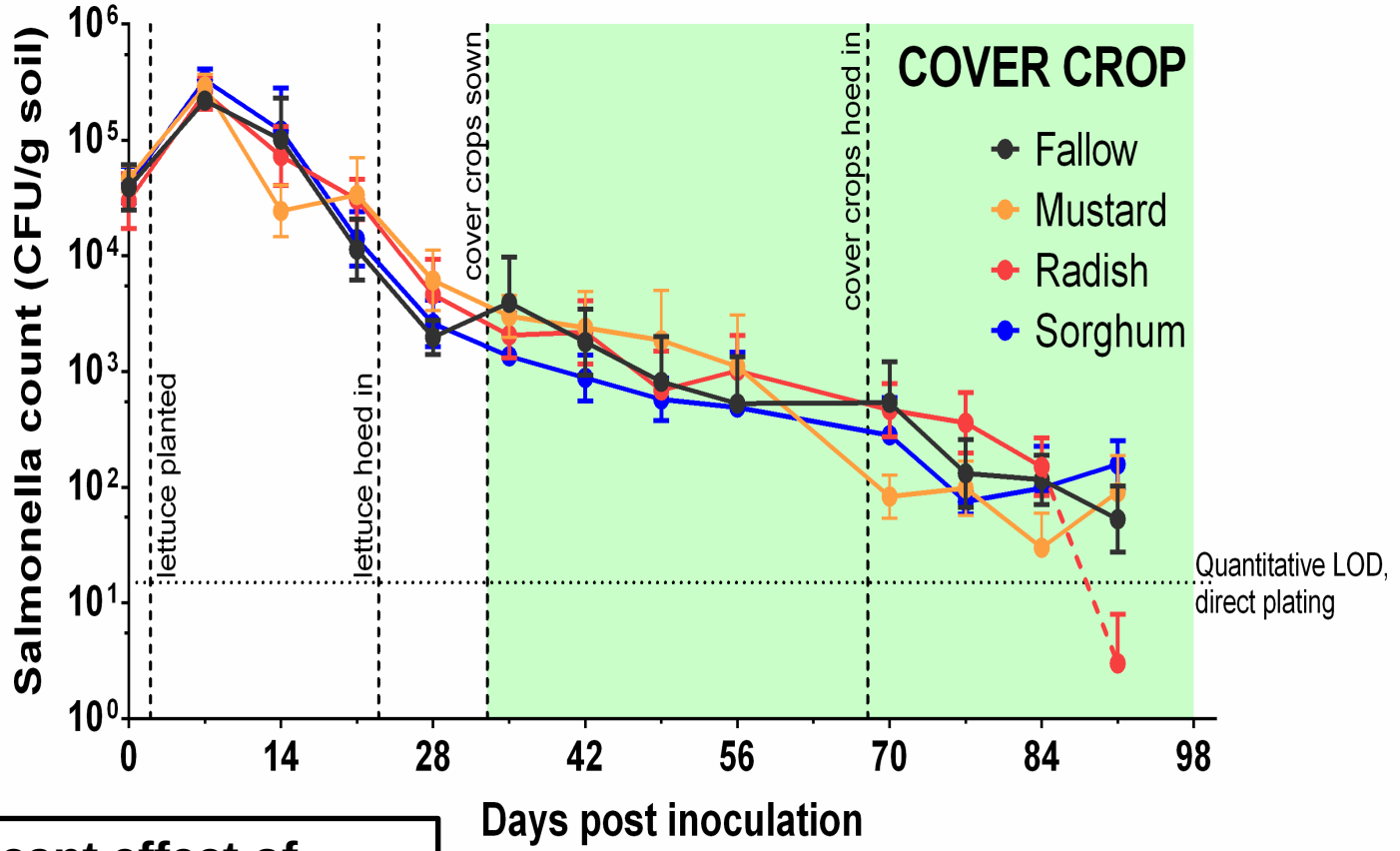
Turn in Lettuce

Cover Crops

Turn in Cover Crops

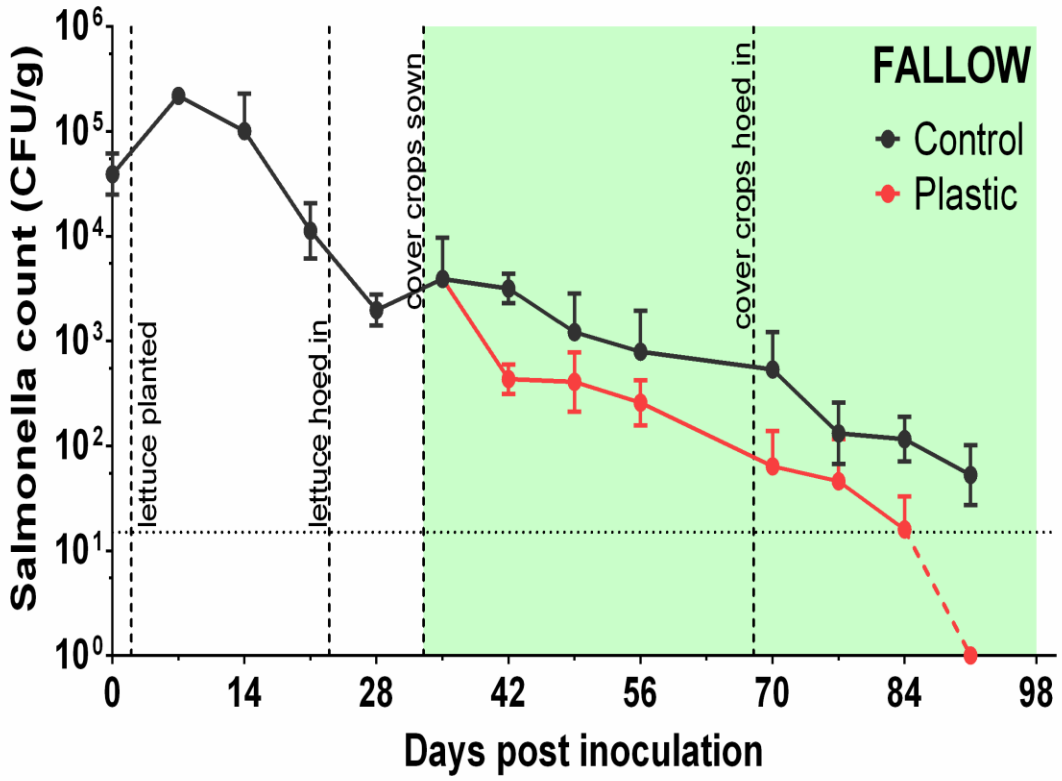
Solarisation

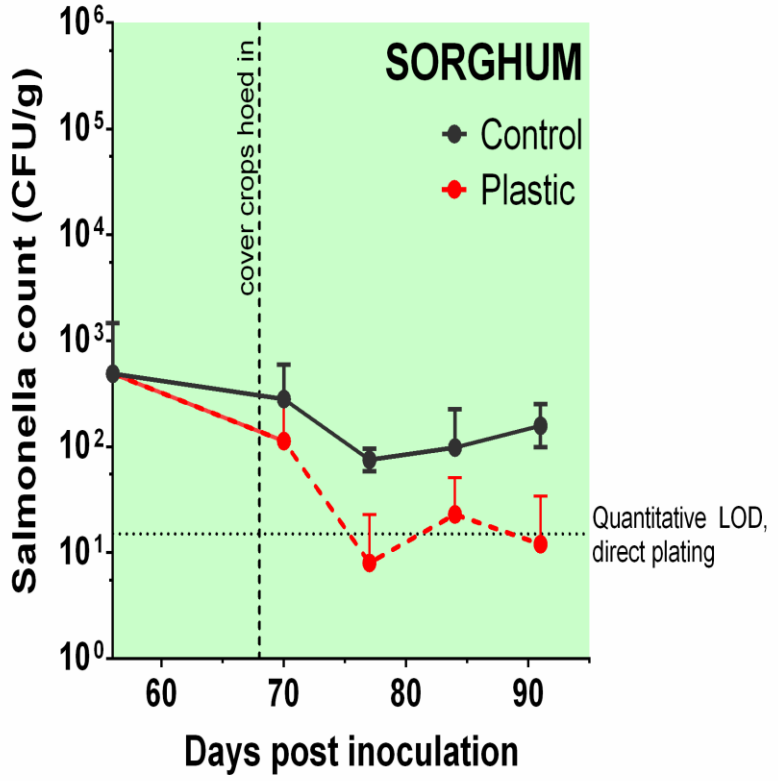
Field Trial: Effect of Cover Crop



No significant effect of cover crop on *Salmonella* survival in clay loam

Field Trial: Effect of Solarisation





Reduced survival of *Salmonella* under solarisation in clay loam

US Mesocosm Trial: Contaminate – Cover Crop – Replant

Contaminate with Chicken Manure

Baby Spinach ‘replant’

US Field Trial:
500 g Inoculated Chicken Pellets Per Plot

Preliminary Conclusions: Pot Study

- Survival of *Salmonella* in sandy soil below detection limit after 21 days
- Survival of *Salmonella* enhanced in clay loam

  (agrees with Brennan et al., 2003)
- 37^0C caused die-off of *Salmonella* in 28 da*ys*
- Addition of manure promotes survival

  (agrees with You et al., 2006)

Preliminary Combined Conclusions: Cover Crop

- US mesocosm trial *Salmonella* survival counts showed promise of reduction by cover crop
- But in AU and US field trials, *Salmonella* survival not affected by cover crop
  - AU: Similar decline *Salmonella* over time for all treatments
  - AU: Levels only reached no detection after 100 days
  - US: Low levels of starting inoculum led to rapid die-off for all treatments
- Effect of cover crop on microbial community and antimicrobial compound analysis yet to be done

Preliminary Combined Conclusions: Solarisation

- Solarisation reduced *Salmonella* survival in both AU and US field trials
  - AU: Significantly reduced recovery compared to control during field trial
  - AU: **0% detection** after 49 days under solarisation but **100%** of enriched samples were positive
  - US: All solarised plots **0% detection** post-enrichment and all fallow controls **100% detection**
- Further statistical analysis and repeat trials are in progress

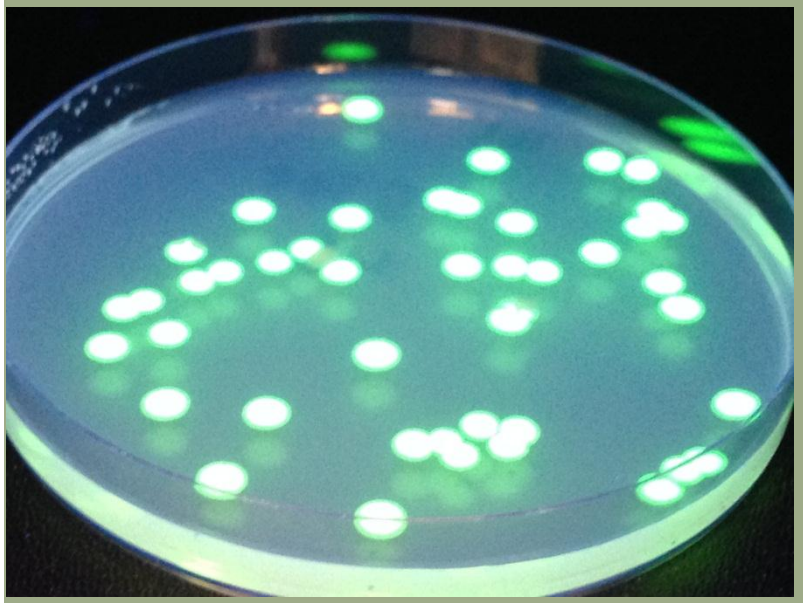
OUTCOMES FOR GROWERS

- Persistence of Australian *Salmonella* serovars in amended and non amended soils
- Potential for risk *L. monocytogenes* in amended and non amended soils
- Efficacy of cover crops and solarisation to expedite die-off of Salmonella in contaminated soils
- Best practice re-plant of vegetables in previously contaminated soils
- Best practice guidelines for safe use of chicken manure amendments.

Acknowledgements

› Funded by Horticulture Innovation Australia Limited with co-investment from the University of Sydney and HIA funds sourced from the Vegetable Industry Levy and the Australian Government and the Centre for Produce Safety

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



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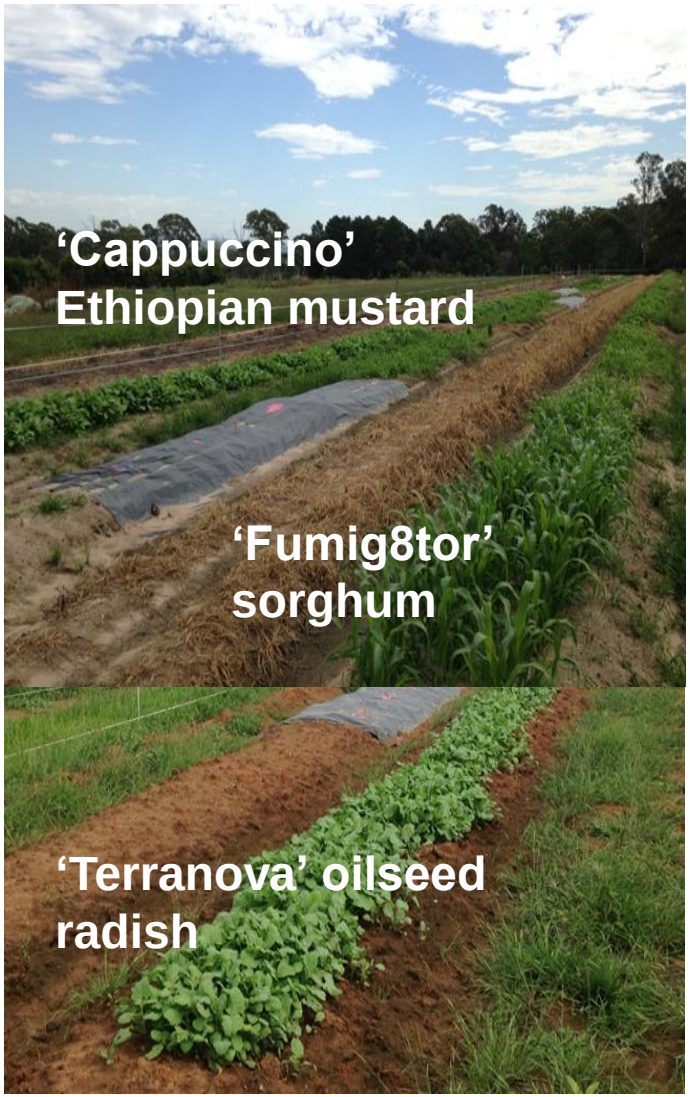
- Australia – most salad producers do not use manure amendments because of the risk
- In US use of heat-treated chicken manure pellets (standards)

FPSC and CPS collaboration

- Priority for US and Australia to understand the risks and look at methods to remediate soils contaminated with *Salmonella*
- Collaboration with the US Center for Produce Safety and Australian vegetable industries through HIA
- UC Davis and University of Sydney
- Similar protocols in two continents
- Reporting February 2016

Project aims



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  - *Salmonella* recovery under controlled conditions
  - Soil type, manure, temperature
- **Mesocosm trial (US)**
  - *Salmonella* recovery under field conditions at a small scale
  - Cover crop
- **Field trials (Australia and US)**
  - *Salmonella* recovery under field conditions
  - Soil type, cover crop, solarisation
  - Presence absence *L.monocytogenes*

Pot study: soil type, temperature, ± manure

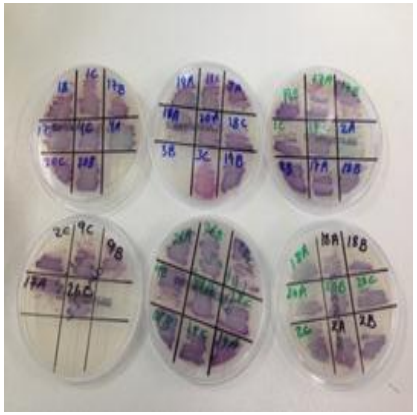
Inoculate with *Salmonella*

Incubate

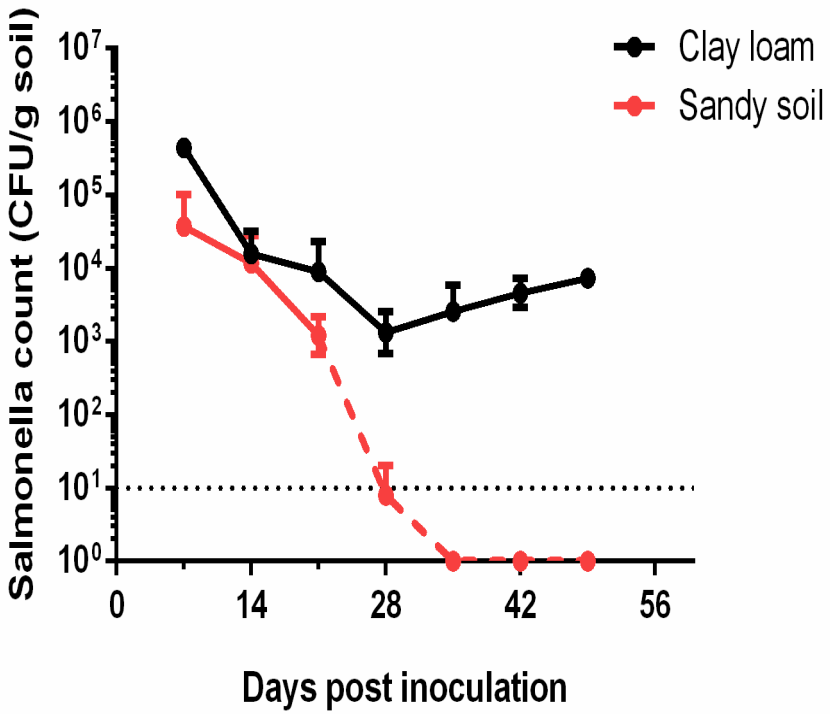
Extract

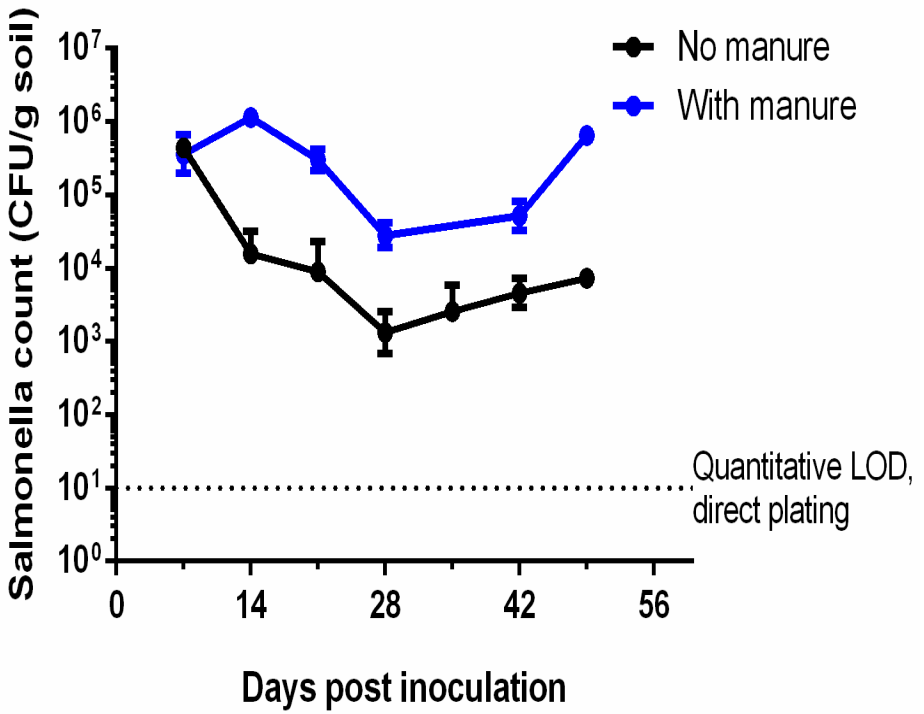
Enumerate

Enrichment



Pot study: *Salmonella* cocktail recovery over time

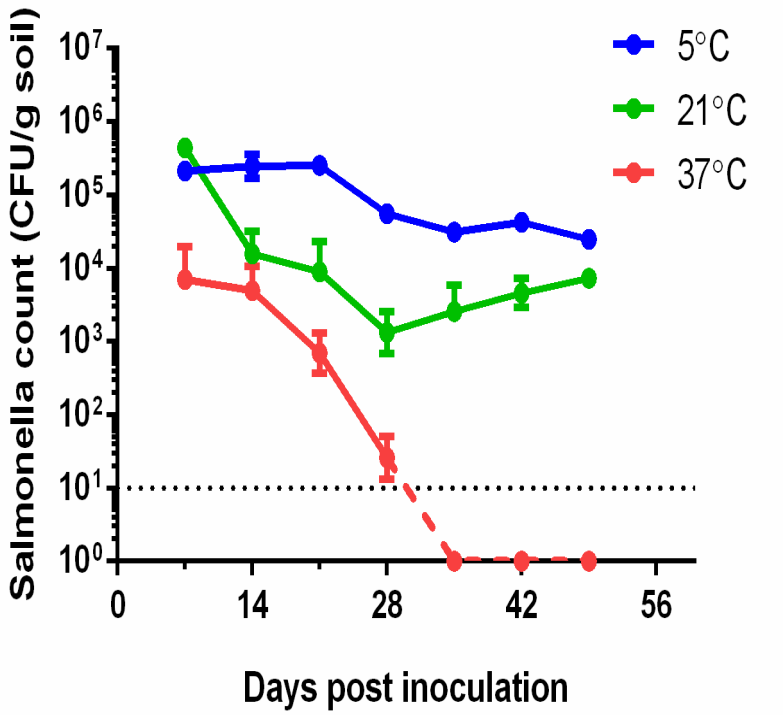


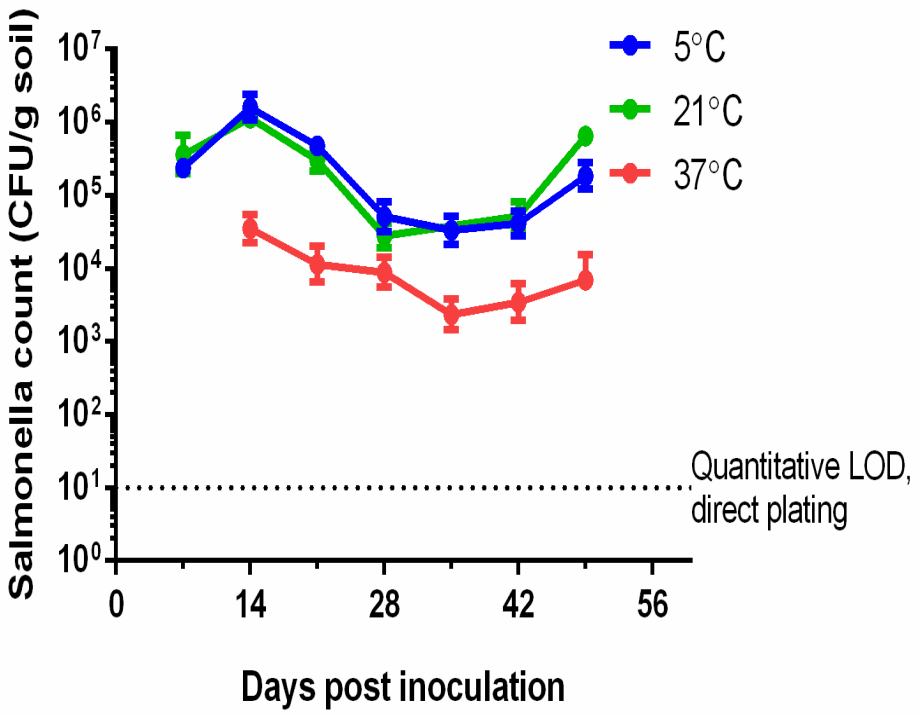


Clay loam promotes survival of *Salmonella* compared to sandy soil

Presence of manure promotes *Salmonella* survival

Pot study: *Salmonella* cocktail recovery at 5, 21, 37 ºC





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Inoculate Manure

Manure Application

Plant Lettuce

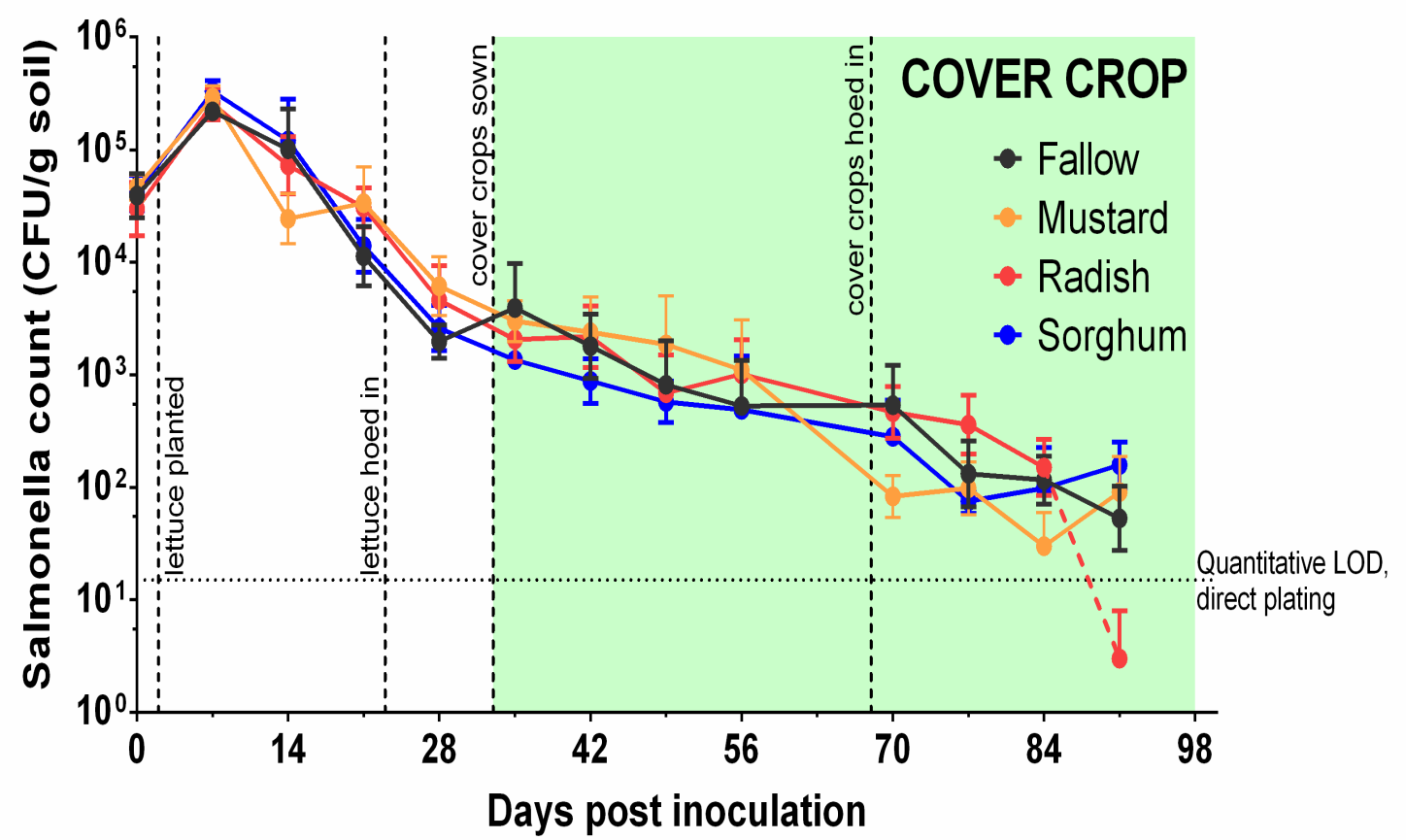
Turn in Lettuce

Cover Crops

Turn in Cover Crops

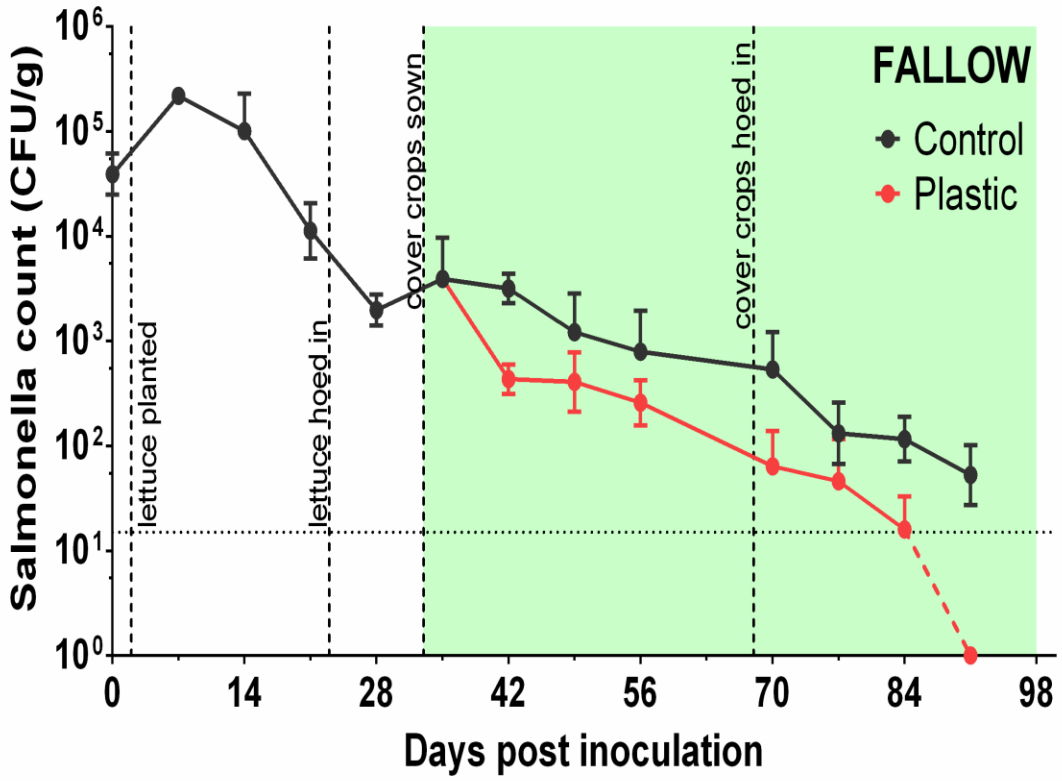
Solarisation

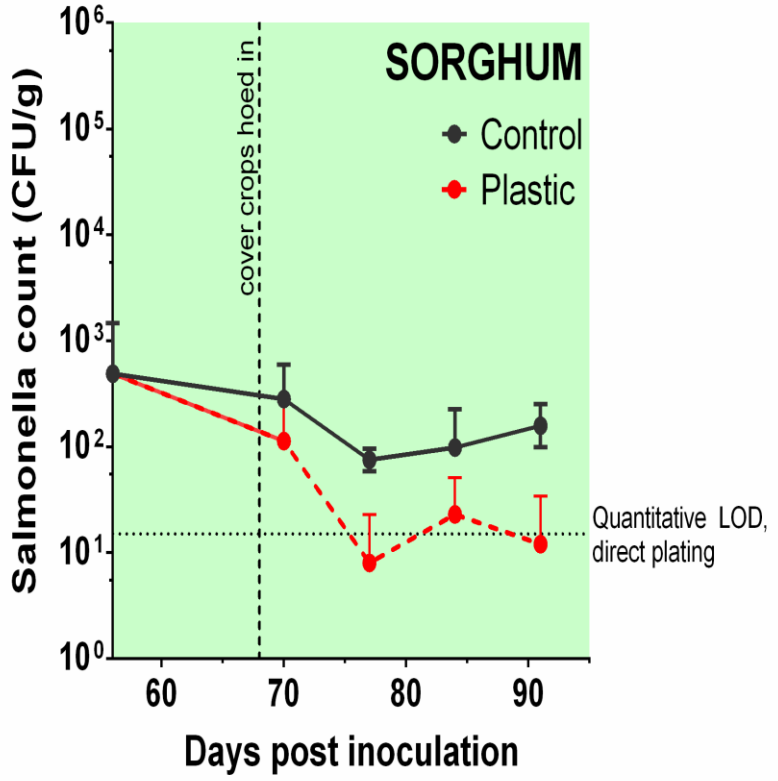
Field trial: effect of cover crop



No significant effect of cover crop on *Salmonella* survival in clay loam

Field trial: effect of solarisation





Reduced survival of *Salmonella* under solarisation in clay loam

US mesocosm trial: Contaminate – cover crop – replant

Contaminate with Chicken Manure

Baby Spinach Replant

US field trial:
500 g inoculated chicken pellets per plot

Preliminary conclusions: pot study

- Survival of *Salmonella* in sandy soil below detection limit after 21 days

- Survival of *Salmonella* is enhanced in clay loam

  (agrees with Brennan *et al*., 2003)

- 37 ºC caused die-off of *Salmonella* in 28 days

- Addition of chicken manure promotes survival

  (agrees with You *et al*., 2006)

Preliminary combined conclusions: cover crop

- US mesocosm trial *Salmonella* survival counts showed promise of reduction by cover crop
- But in AU and US field trials, *Salmonella* survival not affected by cover crop
  - AU: Similar decline *Salmonella* over time for all treatments
  - AU: Levels only reached no detection after 100 days
  - US: Low levels of starting inoculum led to rapid die-off for all treatments
- Effect of cover crop on microbial community and antimicrobial compound analysis yet to be done

Preliminary combined conclusions: solarisation

- Solarisation reduced *Salmonella* survival in both AU and US field trials
  - AU: Significantly reduced recovery compared to control during field trial
  - AU: **0% detection** after 49 days under solarisation but **100%** of enriched samples were positive
  - US: All solarised plots **0% detection** post-enrichment and all fallow controls **100% detection**

- Further statistical analysis and repeat trials are in progress

Outcomes for growers

- Persistence of Australian *Salmonella* serovars in amended and non-amended soils
- Potential for risk *L. monocytogenes* in amended and non-amended soils
- Efficacy of cover crops and solarisation to expedite die-off of *Salmonella* in contaminated soils
- Best practice re-plant of vegetables in previously contaminated soils
- Best practice guidelines for safe use of chicken manure amendments

Acknowledgements

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