Hort Innovation

Final Report

Pathogen persistence from paddock to plate

Project leader:

Emma Walters

Delivery partner:

Fresh Produce Safety Centre

Project code:

VG16042

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Disclaimer:

Funding statement:

This project has been funded by Hort Innovation, using the Vegetable research and development levy and contributions from the Australian Government. Hort Innovation is the grower-owned, not-for-profit research and development corporation for Australian horticulture.

Publishing details:

ISBN 978 0 7341 4614 4

Published and distributed by: Hort Innovation

Level 7
141 Walker Street
North Sydney NSW 2060

Telephone: (02) 8295 2300

www.horticulture.com.au

Content

Public Summary

Consumers naturally expect that food they eat will not make them sick. Bacteria that cause illness, such as *E. coli* and *Salmonella*, can be present in manures added to soil or carried in irrigation water. It is critically important to prevent these human pathogens contaminating leafy vegetables.

In this study, a survey showed that human pathogens were rarely found on Australian fresh vegetables. Pathogenic bacteria were found on less than 1% of 5,533 samples, with most at levels unlikely to cause illness. Bacteria were also uncommon in samples of manure/compost used on farms and in irrigation water.

Trials examined pathogenic die-off rates of bacteria added to poultry litter or cattle manure and incorporated into soil used to grow lettuces. Under these conditions, the average population of *E. coli* in soil plus poultry litter fell by >99% within 20 days. In soils amended with cattle manures, *E. coli* also declined rapidly in two trials in spring and summer, however populations persisted during autumn. The data indicate that *E. coli* was generally reduced to below or close to detectable levels within 50 days, while in some cases *Salmonella* spp. could persist in soils up to 60 days. Three of 200 lettuces were contaminated with *E. coli* at harvest, and none positive for *Salmonella* spp. High temperatures, dry conditions and other environmental factors reduce survival of human pathogens in soil, but effects are variable. Withholding periods between application of manure and harvest provide one method to reduce risk, but the length of withholding periods used will vary according to environmental or growing conditions.

Trials also examined how quickly bacteria died on lettuce leaves after irrigation with contaminated water. *E. coli* and *Salmonella* were undetectable after two days on intact vegetables, but could survive at least six days on damaged lettuces. Even damage occurring four days before irrigation increased survival of *E. coli* on lettuce. If water quality is poor or unknown, a 48-hour withholding period between irrigation and harvest significantly reduces the risk that vegetables will be contaminated at harvest. However, longer withholding periods are needed if plants have been physically damaged.

Summary

Consumers naturally expect that the food they eat will not make them sick. Maintaining a high standard of food safety is critically important for products that are eaten uncooked, such as leafy salad greens. This project has examined factors that potentially affect contamination of vegetables by human pathogens. This included benchmarking surveys of examining what human pathogens are present on fresh vegetables, in irrigation water, in manure and in soil amendments used by growers. Trials examined die off rates of human pathogens in manure amended soils and on leaves after irrigation with contaminated water.

Test results from 5,533 samples of fresh vegetables were collated from all vegetable growing regions of Australia. Overall, less than 1% of tests for *Escherichia coli*, *Salmonella* spp. or *Listeria* spp. were positive. There were no detections of *Salmonella* spp. from 4,707 tests for this bacteria.

Poultry litter and cattle/dairy manure were found to be the two main products used on vegetable farms. Although human pathogens are common in these manures at source, they were rarely detected in soil amendments used at vegetable farms. This suggests that populations of human pathogens often decline significantly by the time manures are incorporated into soil. Moreover, while 20% of the accessed irrigation water test results were positive for *E. coli*, only 5% contained >100 CFU/100ml.

Poultry litter and cattle manure inoculated with non-pathogenic *E. coli* plus *Salmonella* sofia or *Listeria innocua*. were incorporated into soil in three trials conducted in spring (A), summer (B) and autumn (C). Pathogen counts in soils were highly variable. The mean population of *E. coli* in soil plus poultry litter fell from log 3-4 CFU/g to below 10 CFU/g within 20 days of application in all trials, although occasional high (>100 CFU/g) readings continued for up to 40 days. Results in soil plus cattle manure were similar in trials A and B. However, *E. coli* proved more persistent in trial C. The data was modelled using a poisson regression model. Predicted populations of *E. coli* in soil after 50 days were at or below the level of detection across all treatments and seasons, equivalent to 99.9% mortality. *Salmonella* populations also fell rapidly after manure application. Modelling of *Salmonella* (detected/not detected) as binary data indicated that after 50 days the probability of a positive detection was 0.44, 0.1 and 0.02 in spring, summer and autumn trials respectively. *Listeria,* a natural soil dwelling organism, remained detectable for 50 days in spring and autumn trials.

Survival of *E. coli* and *Salmonella* spp. applied to plants through contaminated water was strongly affected by damage. If plants were intact, bacteria fell below detectable levels within 2 days of application. However, damage increased survival, both *E. coli* and *Salmonella* spp. persisting for at least 6 days on injured cos lettuce.

Survival of human pathogens on spinach was only increased when the plants were damaged an hour before contamination. While the highest populations of pathogens on lettuce were found on lettuce damaged one hour before contamination, damage 4 days earlier also significantly increased pathogen persistence. It is concluded that even slight damage due to physical injury or disease may allow human pathogens in irrigation water to persist on leafy vegetables, with cos lettuce particularly at risk.

High temperatures, dry conditions and other environmental factors reduce survival of human pathogens in soil, but effects are variable. Withholding periods between application of manure and harvest provide one method to reduce risk, but the length of withholding periods used will vary according to environmental or growing conditions.

If water quality is poor or unknown, a 48-hour withholding period between irrigation and harvest significantly reduces the risk that vegetables will be contaminated at harvest. However, longer withholding periods are needed if plants have been physically damaged.

Keywords

Food safety; Vegetable; E. coli; Listeria; Salmonella; Irrigation water; Manure; Pathogen

Introduction

In 2015 the Fresh Produce Safety Centre commissioned the project 'Understanding the Gaps', to summarise current fresh produce food safety knowledge and identify research needs. This review addressed priorities that were initially identified by industry and food safety trainers and auditors and refined by industry participants at the 2014 Fresh Produce Safety Centre Food Safety Conference. The outcome was a set of key recommendations on issues needing more research. These included:

1. What is the background level of microbial contamination of fresh produce in Australia?
2. What is the potential for pathogen transfer from agricultural water to product surfaces? How well can pathogens survive and grow on fresh produce in different environments (pre-harvest and postharvest) and what factors increase or decrease survival?
3. What is the potential for pathogen survival in different types of manure or other untreated materials of animal origin placed on, or incorporated into, soil? How is this affected by soil type and what is the subsequent survival on harvested parts of fresh produce?

The risk of contamination of fresh produce with human pathogens from irrigation water and soil amendments is usually managed through setting withholding periods that define the time between application and harvest.

The Guidelines for Fresh Produce Food Safety (2019) stipulate a withholding period of 48 hours between pre-harvest water use and harvest if water contacts the harvestable part of products that may be eaten uncooked (e.g. overhead irrigation or spray application to leafy salad greens).

The Guidelines also propose a minimum 90 day exclusion period between application of untreated soil amendments and harvest if the harvestable part is grown in or close to the soil and may be eaten uncooked (e.g. carrots, leafy greens) and a 45 day exclusion period for lower risk products (e.g. potatoes).

These exclusion periods are based on published data. However, most research has been conducted in the USA or Europe, where temperatures, UV radiation and soil types are very different to Australian conditions. Moreover, studies often use initial concentrations of pathogens higher than would be expected to occur through normal commercial practices.

The issue of soil amendments has become particularly significant as some food safety standards have moved to mandate 365 day withholding periods on use of all materials of animal origin (unless treated and certified as meeting AS4454). This would effectively make these products unworkable for vegetable farmers.

At the same time, recent outbreaks of food-borne pathogens (e.g. *Listeria monocytogenes* on melons) has sharpened interest in understanding and mitigating sources of risk.

This project has examined the population dynamics of human pathogens in manure amended soils and carried in contaminated irrigation water. As with all microbial studies, the response of populations will vary based on individual climatic variables and production practices, however this may provide valuable evidence as to the appropriateness of current recommendations on withholding periods in Australia as part of a holistic approach to managing food safety.

Methodology

Determine existing levels of food safety pathogens on Australian vegetables

A very large data set was collated, with results from 5,533 individual samples of fresh, levy-paying vegetables. Data was provided by numerous contributors, including individual businesses, FreshTest and QA service organisations. While many samples were submitted for testing by individual companies, others were randomly selected from wholesale markets. While this sampling method is not perfect, it seems unlikely that business QA staff would deliberately submit samples less likely to have microbial contamination, so samples are likely to reflect products destined for retail.

Records were sourced from all vegetable production areas of Australia, providing a good overview of vegetable production nationally. Entries were manually coded according to region, season (spring, summer etc.) as well as product category, product type (leaf, stem, root etc), food safety risk (high or low), type of detection and level (CFU/g).

More information on the methodology of this survey is included in Appendix 1.

Conduct a survey of grower practices

Freshcare staff emailed 1,191 vegetable growers to request participation in the survey of grower practices with regard to composts and manures. 41 responses were received, of which 14 indicated that they did not use any of these products.

The remaining 27 growers were then individually contacted by phone and interviewed regarding their use of manures using a semi structured discussion plan. Questions included the products used, how often and in what quantities they were applied, and what crops were grown. While it had originally been planned to discuss the issue with additional growers, little new information was received after 27 interviews so the activity was terminated.

Determine background levels of human pathogens in manure

Review of literature

A brief review of international literature on human pathogens was conducted. Sources included Australian data on poultry litter and cattle manure (from the literature and directly from Australian researchers), as well as international reviews with data from countries as diverse as USA, Europe, China, Canada and Turkey. Average populations of key pathogens and the range that could be expected were tabulated.

Sampling from vegetable farms

As previously noted, use of manures is a sensitive subject among vegetable growers, which made it difficult to obtain samples directly from farms. In total, 41 samples were collected and submitted to Symbio laboratory for testing. Each sample was tested for *E. coli* (CFU/g), while *Salmonella* spp. and *Listeria* spp. were recorded as Detected/Not Detected in 25g using the VIDAS method, with confirmation through plating.

Determine background levels of human pathogens in irrigation water

Over 4,000 water test records were collated. However, 3,800 of these records were sourced from a laboratory in Western Australia; the large number of different laboratories used on the east coast meant that, unlike WA, this information did not have a central repository. Inferences from this data are also limited by the fact sampling was not random, with most samples likely to have been submitted to meet audit requirements.

The data were compared with results from independent samples collected from the Hawkesbury region by University of Sydney PhD candidate Ms Emily White. The results were also compared with international data on background levels of pathogens in irrigation water.

More information on the methodology of these surveys is included in Appendix 2.

Determine pathogen survival times in different types of manure amended soil

Trial design

Three trials were conducted (spring (A), summer (B) and autumn (C)) using forty 2m x 1m boxes located at the University of Sydney field site at Cobbitty. Each was dug into the surrounding soil approximately 20cm, then filled to 30cm depth with either a sandy or clay loam soil, these being typical of those used to grow vegetables in the region. Soil moisture probes were installed in a number of the boxes and a weather station placed in the centre of the trial block.

The 20 boxes/soil type were randomly allocated to one of five treatments:

1. Unamended control
2. Poultry litter
3. Cattle manure
4. Poultry litter + log 5 CFU/g *E. coli* + log 3.4 CFU/g *Salmonella* sofia
5. Cattle manure + log 5 CFU/g *E. coli* + log 3.4 CFU/g *Listeria innocua*

Manures were added to each box at a rate of 20t/ha (4kg/box). This is higher than the usual commercial application rate of 5-10t/ha, but not implausible.

Two of the treatments involved artificially inoculating the manures as initial sampling did not detect any human pathogens. Cultures containing approx. 9 CFU/ml of the target organisms were prepared by University of Sydney staff. Serial dilutions were conducted for each box, with final dilution into 2L of reverse osmosis (RO) filtered water which was mixed into the 4kg manure sample. Manure was then evenly incorporated into the top 5-8cm of soil.

Figure 1. Inoculation of manure with human pathogens, and addition to boxes

Each box was planted with 27 cos lettuce seedlings six days after the manure was added, providing a density similar to a commercial bed. Plants were irrigated with overhead sprinklers with the aim of maintaining at least 30% moisture in clay or 20% moisture in sandy soil. Plant size and colour were recorded at commercial maturity.

Figure 2. Lettuces at planting (left) and commercial maturity (right)

Microbial testing

All microbial testing of soil, manure, water and plants was conducted by Symbio Laboratory North Ryde, which is NATA accredited for these analyses. Methods used were;

E. coli	Enumeration by petrifilm (M8.8, reference method AOAC 991.14), limit of detection of 10 CFU/g
Salmonella spp.	Detection by VIDAS (M16.4, AOAC RI approved protocol no. 071101) in 25g of sample. Positive results confirmed by MALDI-ToF and *Salmonella* serology (MALDI ToF AOAC-OMA 2017.09 and AS5013.10). Population estimated using a 3-tube serial dilutions with most probable number (MPN) technique.
Listeria spp.	Detection by VIDAS (M13.4) in 25g of sample. Positive results confirmed by MALDI-ToF and *Listeria* serology AFNOR Bio-12/33-05/12.

At the start of each trial samples were taken of irrigation water, manures, inoculants and inoculated manures. Soil samples were taken from each box immediately after incorporation of the manures, when the lettuces were planted, then at intervals until the lettuces reached full commercial maturity. In the summer and autumn trials whole lettuces were also sampled from each box with outer leaves and soil attached. To allow graphical representation of the data, a value of "9" was used where *E. coli* was <10 CFU/g, and data was normalized by transforming to log 10 values.

Data was modelled using count data for *E. coli* populations in soil. A poisson regression model was applied, with fixed factors for manure, season and soil type. *Salmonella* was modelled as binary data (detected/not detected) using a logistic regression model. Analysis of variance was used to assess differences between harvested lettuces.

More information on the methodology of these trials is included in Appendix 3.

Determine potential for pathogens to transfer from irrigation water to product surfaces and factors affecting survival

A series of trials were conducted examining die off rates of *E. coli* and *Salmonella* spp. on the surfaces of leafy vegetables including cos lettuce, baby spinach, parsley, kale and silverbeet (chard). The plants were damaged or left intact before irrigation to runoff with contaminated water. A summary of the trials is shown in Table 1.

Table 1. Trials conducted examining persistence of *E. coli* and *Salmonella* spp. on vegetables after irrigation with contaminated water

Trial	Start date	Aim	Method
T1 T2 T3	30/10/18 13/11/18 27/11/18	To test persistence of *S. sofia* and *E. coli* on vegetables.	Kale, silverbeet (chard), parsley and cos lettuce grown in pots in a glasshouse then transferred to outside just before treatment. Plants irrigated with water containing log 3 CFU/ml *E. coli* + log 2 CFU/ml *S*. sofia. Plants tested for up to 6 days.
T4a T4b	8/1/19 15/1/19	To test persistence of *S. sofia* and *E. coli* on damaged vs undamaged vegetables.	Cos lettuce, parsley and chard were grown in pots in a glasshouse. Cos lettuce was left intact or slightly damaged by gently squeezing the leaves. Plants irrigated with water containing log 3 CFU/ml *E. coli* + log 2.3 CFU/ml *S*. sofia in-situ. Plants tested for up to 6 days.
T5a T5b	9/4/19 15/4/19	To test persistence of *S. hofit* and *E. coli* in a field situation using cos lettuce (damaged and undamaged), parsley and babyleaf spinach.	Baby spinach, parsley and cos lettuce were grown outside in raised boxes at Cobbitty. Cos lettuce was left intact or slightly damaged by clipping the core leaves. Plants irrigated with water containing log 3 CFU/ml *E. coli* + log 2.3 CFU/ml *S*. sofia. Plants tested for up to 6 days.
T6a	11/10/19	To examine the effect of delay	Baby spinach and cos lettuce were grown

T6b T6c T6d	18/10/19 1/11/19 8/11/19	between damage and irrigation with contaminated water on persistence of *S. hofit* and *E. coli* on cos lettuce and baby spinach.	outside in raised boxes at Cobbitty. Plants were protected from pests using suspended netting. Damage was inflicted 96 or 72, 48, 24 or 1 hour before irrigation with water containing log 3.5 CFU/ml *E. coli* + log 2.5 CFU/ml *S. hofit*. Plants tested immediately and after 48 hours.

More information on the methodology of these trials is included in Appendix 4.

Outputs

Outputs from the project have so far been limited due to the preference for peer review before promotion of the results to industry members. Two papers are now under preparation and will be submitted for peer review:

- Differences in persistence of *Salmonella hofit* and *Escherichia coli* on cos lettuce compared to babyleaf spinach
- Persistence of human pathogens in manure amended Australian soils used for production of leafy vegetables

The abstracts of these two papers are included as Appendix 5.

Despite this limitation, a number of communication activities have occurred:

- "How Safe are my Soils" – Article in Vegetables Australia magazine, October 2018
- "How Safe are my Soils" – Article in WA Grower, spring 2018
- "Pathogens on leafy greens" – Presentation to the Fresh Produce Safety Centre conference, August 2019
- "Managing the risk from microbes on leafy greens" – Presentation to management committee of HARPS (included representatives from Coles, Woolworths, Aldi, Costco, Metcash and McDonalds)
- "Horticultural water and food safety" – Presentation to International Commission on Microbiological Specifications for Foods, October 2019
- "Guidelines for Fresh Produce Food Safety" – Presentation to APAL conference, January 2020
- "Managing the risk from microbes on leafy greens" – Presentation to Stanthorpe vegetable growers group, February 2020

Project Summaries / Fact Sheets have been prepared. These will be promoted and made available through the Fresh Produce Safety Centre and Freshcare, once approved by HIA. The papers will be submitted to a special issue of *Agriculture*, focusing on "Quality and Safety of Fresh Produce", with guest editor and Board member of the FPSC, Professor Robyn McConchie. Submissions for this journal are due in November 2020.

These project summaries/fact sheets are included as Appendix 6.

Outcomes

Determine existing levels of food safety pathogens on Australian vegetables

Less than 1% of tests detected any type of human pathogen. Moreover, despite over 4,700 tests for *Salmonella* spp., there were no detections of this bacteria.

Table 2. Summary of test results

Test conducted	Number of samples tested	Total positive samples (%)
E. coli	5,387	1.8
Salmonella spp.	4,707	0.0
Listeria spp.	1,195	1.6
L. monocytogenes	2,488	0.2

Low populations of both *Listeria* spp. and *Listeria monocytogenes* were detected. Of a total of 3,683 tests conducted for these two organisms, 23 were positive. The highest enumerated results were 50 CFU/g for *Listeria* spp. and 5 CFU/g for *L. monocytogenes*.

The majority (85 of 117) of positive detections were of *E. coli*. Most of these were at low levels (less than 100 CFU/g), as shown in Figure 3. According to FSANZ Guidelines[i], <100 CFU/g *E. coli* is acceptable for ready to eat foods, although clearly not desirable.

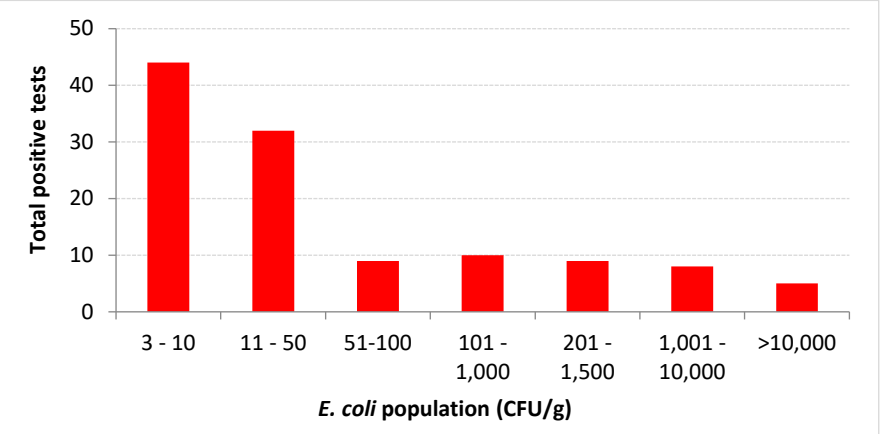

Figure 3. Total number of tests that recorded a positive detection for E. coli, grouped by the result of enumeration.

A number of trends were observed:

- Leafy vegetables had more microbial detections than fruiting vegetables, likely due to their large surface area relative to volume, textured surface, proximity to soil and irrigation close to harvest.
- There were more positive test results from southern growing areas (Clyde, Gippsland, Werribee, Tasmania) than northern growing regions (Figure 4).
- Vegetables grown close to the soil and which could be eaten uncooked i.e. high risk products, tested positive for *E. coli* slightly more frequently than low risk vegetables.
- Detections of *E. coli* peaked in summer, continuing at high levels into autumn, while *Listeria* spp. detections were highest during autumn.

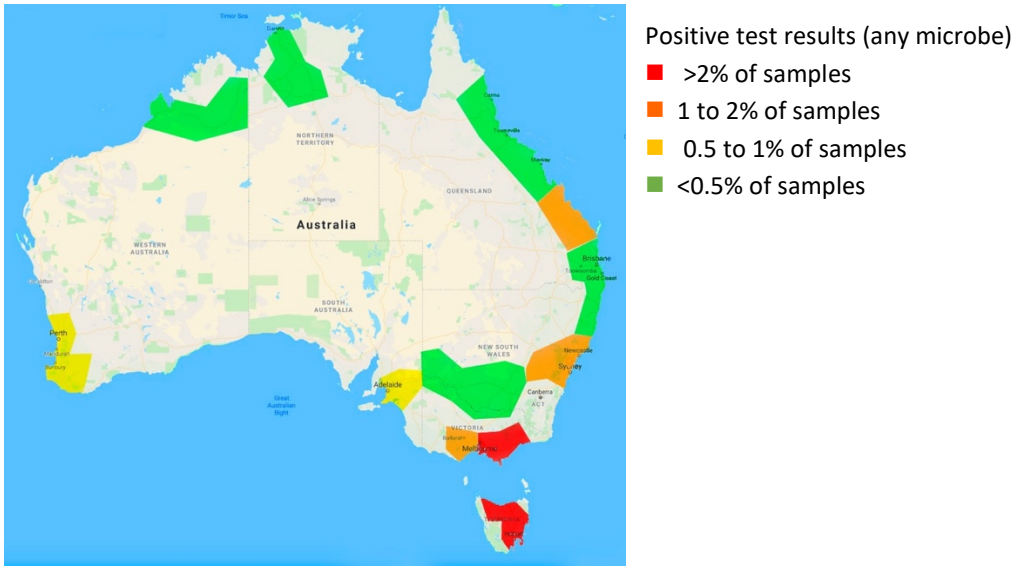

Figure 4. Vegetable growing regions of Australia, coloured according to the frequency of detections of any microbe on fresh vegetables submitted for testing

Detailed results in Appendix 1.

Conduct a survey of grower practices

All growers who were interviewed for the survey were aware of the food safety issues potentially associated with the use of untreated manures. All stated that they were using these products in accordance with Freshcare requirements.

A range of organic materials were nominated, including broiler litter (fresh and composted), cattle manure, pig manure and stable waste. A number of growers indicated they used compost. However whether this material had been treated in accordance with AS4454 was unclear and on-farm composting is unlikely to be certified to this standard.

Determine background levels of human pathogens in manure

The review of International literature demonstrated that fresh manures do not always contain detectable human pathogens, and that pathogens vary between manure types. For example:

- Poultry manure is a key source of *Salmonella* spp., but incidence is highly variable[ii]
- Fresh poultry manure (sampled straight from the shed or the chicken) often contains *E. coli* but human pathogenic strains are rarely isolated[iii]
- Cattle (and sheep) manure is the major source of human pathogenic (Shiga Toxin) *E. coli*, with 6 to 30% of samples containing STEC strains[iv]
- STEC *E. coli* appears to be less common in adult dairy cattle[v] being most prevalent in young animals especially if grain fed[vi]
- Cattle manure can also contain *L. monocytogenes* (5 to 40% of samples) and *Salmonella* spp. (10 to 30% of samples) and is more common in dairy than beef cattle[vii].

Twelve Australian studies were reviewed. These also highlighted the high variability in human pathogen populations in manure samples. Although in some cases prevalence was higher than overseas studies, populations found were generally lower. The results are summarised in Table 3.

Table 3. The percentage of positive tests, average population and range of reported populations of human pathogens in Australian manure samples.

	***E. coli* (all)**			***Salmonella* spp.**			***Listeria* spp.**		
	Positive tests (%)	Average (Log CFU/g)	Range (Log CFU/g)	Positive tests (%)	Average (Log CFU/g)	Range (Log CFU/g)	Positive tests (%)	Average (Log CFU/g)	Range (Log CFU/g)
Broiler litter	94	3.2	0.4 - 7.1	66	1.4	0.6 - 5.4	0		
Cattle manure	94	3.7	1 - 7.4	17	–	.05 - 3.4	83	3.3	2.9 - 5.1
Piggery effluent	100	5.0	8.0	31	2.4	.05 - 5.7			

In total, 41 samples of manure were sourced directly from vegetable farms. These had not been composted, but had been aged for variable periods of time. Samples included horse and cattle manure and poultry litter. Each sample was tested for *E. coli* (CFU/g), *Salmonella* spp. (D/ND) and *Listeria* spp. (D/ND), with the following detections:

- Two samples contained detectable populations of *E. coli* (10 CFU/g), of which the highest was log 3.3 CFU/g.
- *Listeria innocua* / *L. welshimeri*, were detected in 6 samples (15%).
- *Salmonella* spp. was not detected in any sample.

Tests at Australian poultry farms indicated that *E. coli* and *Salmonella* spp. populations at the shed averaged log 5.5 CFU/g and 30 MPN/g respectively[viii]. However, once litter is piled and removed from the shed, composting processes begin and pathogen levels fall. *Salmonella* populations in various manures have been found to decrease by log 2-4 CFU/g within 10 days once the materials are piled up for disposal[ix]. Our limited testing is consistent with this observation, *E. coli* populations ranging from log 1 to 3 CFU/g in manure tested on farm. Trials conducted in WA also failed to detect *E. coli* in poultry litter after delivery to vegetable farms[x].

This suggests that – while highly variable[xi] – pathogen levels in manures are likely to have already declined significantly by the time they are collated and transported to vegetable farms.

Determine background levels of human pathogens in irrigation water

There were no detections of *Listeria* spp. in the accessed water data (147 tests). There was one detection of *Salmonella* spp. in pre-harvest water (250 tests). While 20% of the results were positive for *E. coli*, only 5% contained >100 CFU/100ml. The low level of detections in these samples may be contrasted with the surface water samples collected independently by University of Sydney PhD student Ms Emily White. In these samples the maximum *E. coli* population detected was >2,420 CFU/ml, while the median was 79 CFU/100ml.

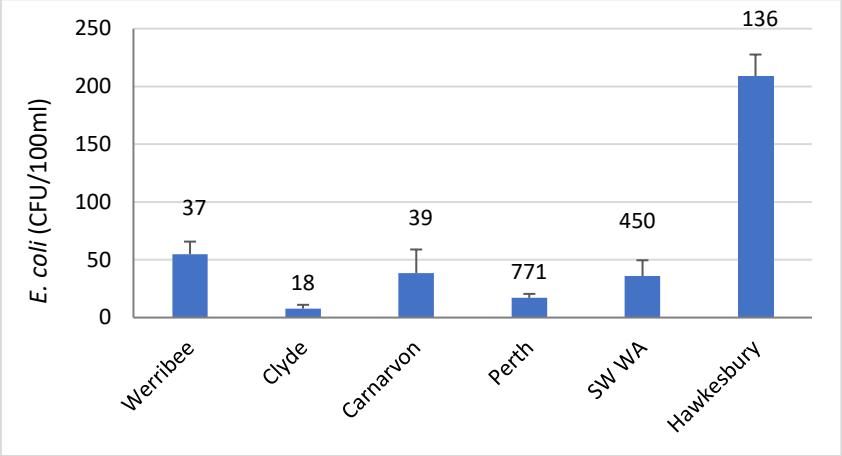

Figure 5. Average populations of *E. coli* in irrigation water used on vegetable farms. Note that only data from the Hawkesbury was independently sampled; other samples were submitted for audit purposes. In all cases the limit of detection was 1 CFU/100ml; 0 was substituted for "not detected". Bars indicate the standard error of each mean value, with "n" indicated above each column.

Determine pathogen survival times in different types of manure amended soil

Initial pathogen levels in manure

Apart from one detection (of nine tests) of *E. coli* in poultry litter there were no detections of *E. coli, Salmonella* spp. or *Listeria* spp. in the unamended manures. Variable results were obtained when manure samples, especially poultry litter, were tested immediately after inoculation with *E. coli* plus *Salmonella* sofia or *Listeria innocua* (Table 4). It appears likely that attributes of the poultry litter used in some trials reduced survival of the bacteria inoculated into the manure.

Table 4. Moisture content and pathogen populations in manures that were left as-is or inoculated with human pathogens for trials conducted in spring (A), summer (B) or winter (C).

		Moisture content (%)	*E. coli* (Log CFU/g)	*Salmonella* sp. (Log CFU/g)	*Listeria* sp. (D/ND)
Poultry litter	A	43	<1	ND	ND
	B	30	<1 to 3.3	ND	ND
	C	26	<1	ND	ND
Poultry litter + *E. coli* + *S. sofia*	A		<1	> 3.0	ND
	B		4.0 to 4.5	1.5 to 3	ND
	C		4.2 to 4.4	1.4 to 3	ND
Cattle manure	A	24	<1	ND	ND
	B	40	<1	ND	ND
	C	36	<1	ND	ND
Cattle manure + *E. coli* + *L. innocua*	A		2.8 to 3.0	ND	Detected
	B		4.7 to 5.2	ND	Detected
	C		5.0 to 5.1	ND	Detected

Climate during trials

Soil moistures were generally consistent across all three trials for the first 30 days of crop growth. However, soil moisture fell significantly as plants approached harvest maturity during both the spring (A) and summer (B) trials. Irrigation was insufficient to replace increasing demands from the growing lettuces as well as high rates of evaporation as temperatures rose.

During the first three weeks, maximum temperatures were relatively mild during trial A (20-25°C), whereas they were hot (25-35°C) and very hot (30-40°C) during C and B respectively. Despite this both trials A and B were characterised by rising temperatures and numerous days with strong solar radiation (>20MJ/m^2). In

contrast, temperatures fell and solar radiation was low during C, with the result soil moisture stayed relatively constant throughout the trial.

E. coli survival in manure amended soils

Differences between soil types were not significant (p=0.176), whereas there were significant differences between manure types (p=0.00), over time (p=0.00) and by trial (p=0.045). Populations of *E. coli* were highest, and had the greatest variance, one week after incorporation into the manure. In both trials A and B, *E. coli* populations in boxes amended with cattle manure fell to close to the level of detection within 10 days. However, the results from autumn (C) provide an important exception. It seems possible that environmental conditions increased persistence of *E. coli*. (Figure 6a).

In the case of poultry litter, a >2 log reduction of the *E. coli* population was observed within three weeks in all trials. However, it should be noted that occasional high readings still occurred 28 (A) and 38 (B) days after inoculation (Figure 6b). Occasional high *E. coli* populations (> log 3 CFU/g) were also recorded in the un-inoculated poultry litter during all three trials. These mainly occurred within the first two weeks of planting, with a trend to more detections in the clay compared to the sandy soil. There were no further detections in these boxes 4 weeks after the start of the trials.

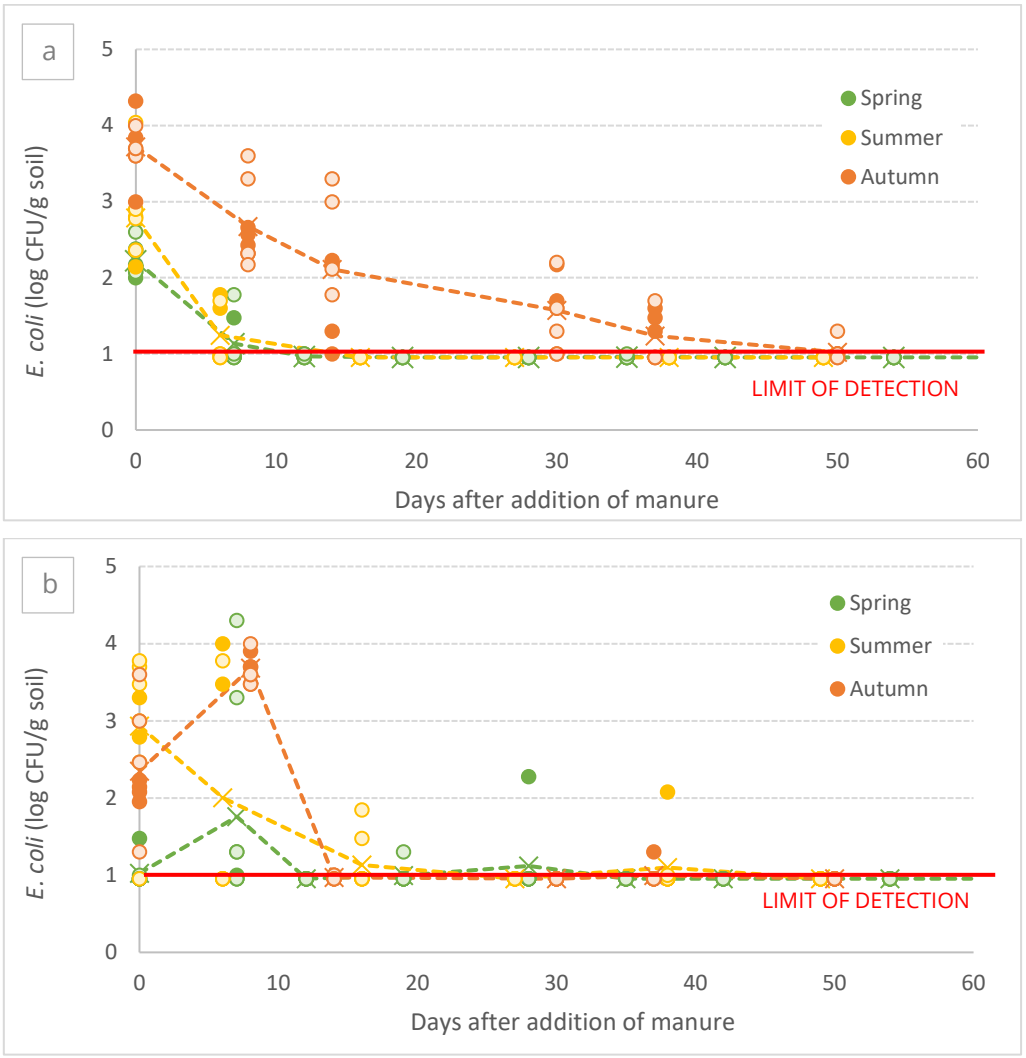

Figure 6. Populations of *E. coli* in sandy (●) and clay (○) soil amended with contaminated cattle manure (a) or poultry litter (b). Each point represents a test result from a single box, giving a total of 4 data points per soil type per sampling time per trial. Dotted lines indicate the mean for each data set.

The data model developed from these results predicted that *E. coli* populations would be at or close to the limit of detection 50 days after manure was added to the soil for all of the different combinations tested

and across all seasons. This suggests that populations are forecast to decline by at least 3 log (99.9%) over this time interval.

Listeria spp. survival in manure amended soils

Listeria spp. was only detected in boxes amended with inoculated cattle manure.

There was no difference in frequency of detection between the sandy and clay soil, so this data was combined. As *Listeria* spp. was not enumerated, results are presented as the proportion of the eight boxes/trial that were positive at each sampling time. While detections decreased over time, it is evident that *Listeria* spp. persisted for more than 50 days in trials A and B. Unlike *E. coli*, *Listeria* is naturally a soil dwelling organism, so this persistence is perhaps unsurprising.

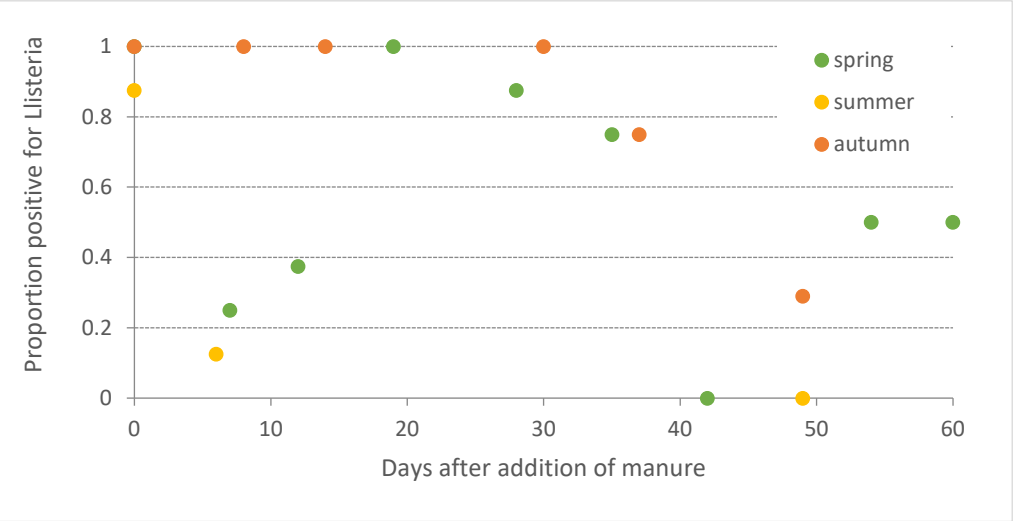

Figure 7. Proportion of boxes (n=8) that returned positive results for *Listeria* spp. after addition of cattle manure contaminated with *L. innocua*.

Salmonella spp. survival in manure amended soils

As with *E. coli*, detections of *Salmonella* spp., and estimates of population from MPN, were extremely variable. To normalize the data, data was converted to logs, with calculations made on the log values. In order to graph the data, a number of assumptions had to be made with test data;

- Where *Salmonella* was detected, but the population was recorded as "<3", a value of "2" (log 0.3 CFU/g) was substituted
- Where *Salmonella* was not detected, a value of "log 0 CFU/g" was substituted
- As the maximum value calculated with MPN was ">1,100", a value of "1,200" (log 3.1 CFU/g) was substituted

Logistic regression of the binary data (detected / not detected) indicated there was a significant difference between seasons and over time. Odds ratios indicated that detection of Salmonella was 5.7 and 41.5 times more likely in trials B and A respectively when compared to trial C. The probability of detection after 50 days was 0.1 and 0.02 in trials B and C respectively. However, after 50 days in spring (A), the probability of detection remained 0.44, indicating a 44% chance of that soil was positive for *Salmonella* spp.

Despite this, populations of *Salmonella* spp. estimated using the MPN method indicated a significant decline in the population over this period. There were no detections of *Salmonella* three weeks or more after manure addition during trials B and C, while populations in trial A declined by nearly 2 log over the trial period (Figure 8).

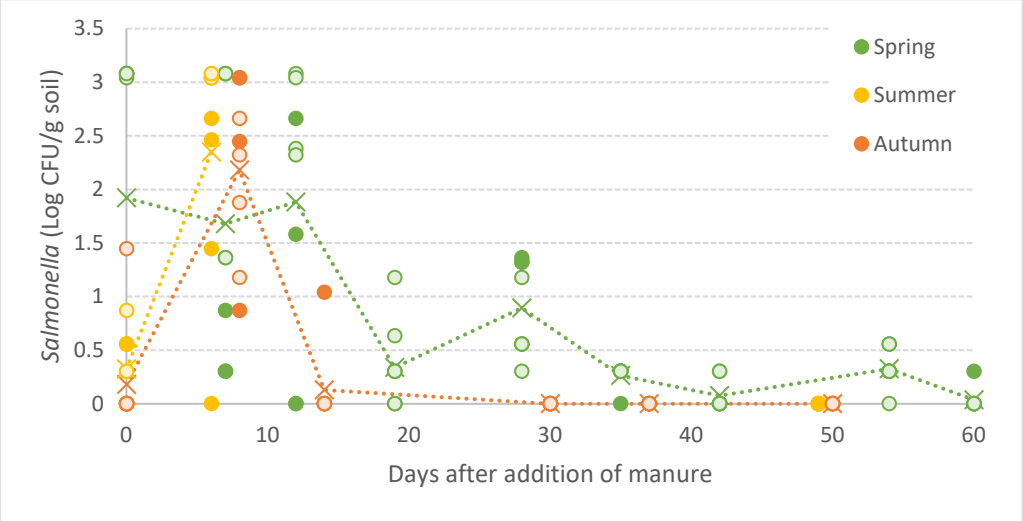

Figure 8. Populations of *Salmonella* spp. in sandy (●) and clay (○) soil after addition of poultry litter contaminated with *Salmonella*. Each point represents a test result from a single box, giving a total of 4 results per soil type per sampling time per trial. Dotted lines indicate the mean for each data set.

Low populations of *Salmonella* spp. continued to be detected 60 days after application of contaminated poultry litter during trial A (spring). However, there were no detections of *Salmonella* three weeks or more after manure addition during B and C. In the case of the autumn (C) trial, this was despite inoculation with additional *S.* sofia 6 days after the initial contamination event.

Pathogen detection on lettuce

There were three detections of *E. coli* >10 CFU/g in trial B and two detections of *Listeria* spp. in trial C. The most significant detection was on a lettuce from an unamended box, which recorded *E. coli* >log 4 CFU/gTable 5.

Table 5. Detections of pathogens on lettuces

Test result	Trial	Days after inoculation	Treatment
250 CFU/g *E. coli*	B	27	Poultry litter
170 CFU/g *E. coli*	B	38	Cattle manure + inoculant
40,000 CFU/g *E. coli*	B	49	Unamended
Listeria – detected	C	29	Cattle manure + inoculant
Listeria – detected	C	29	Cattle manure + inoculant

Lettuce quality and yield

The addition of poultry litter significantly ($p<0.05$) increased the average head weight of lettuces grown in sandy soil in all three trials. In trial A greenness was also increased in these lettuces. Cattle manure had less effect, while differences were generally not significant for lettuces grown in boxes filled with clay loam. In effect, lettuces grown in sandy soil with poultry litter were similar quality to those grown in clay loam regardless of manure addition.

Detailed results in Appendix 3.

Determine potential for pathogens to transfer from irrigation water to product surfaces and factors affecting survival

Trial 1 – Persistence of microbes from irrigation water on vegetables

The weather varied considerably over the three trials conducted.

- A was hot and continuously sunny throughout, reaching 40°C on day 4.
- B was comparatively cool and cloudy, especially for the first 3 days after irrigation.
- C experienced a severe weather event, more than 100mmm of rain falling on the day after irrigation.

Populations of *E. coli* were similar on the contaminated chard, parsley and kale plants, so this data was combined. In contrast, both the number of detections and populations recorded were significantly higher on lettuces.

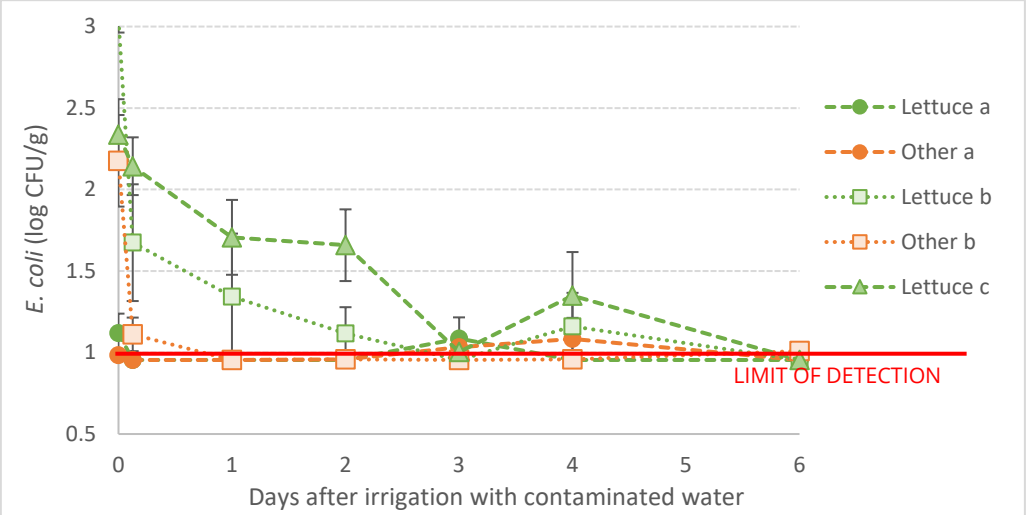

Figure 9. Populations of *E. coli* recorded on lettuces (n=4,8) and on parsley, chard and kale plants (other) (n=12) after irrigation with contaminated water in three separate trials (a to c). Bars indicate the std. error of each mean value.

Salmonella spp. was also detected more frequently on lettuce than the other vegetables. *Salmonella* could still be detected on more than half of the lettuces when tested six days after irrigation with contaminated water.

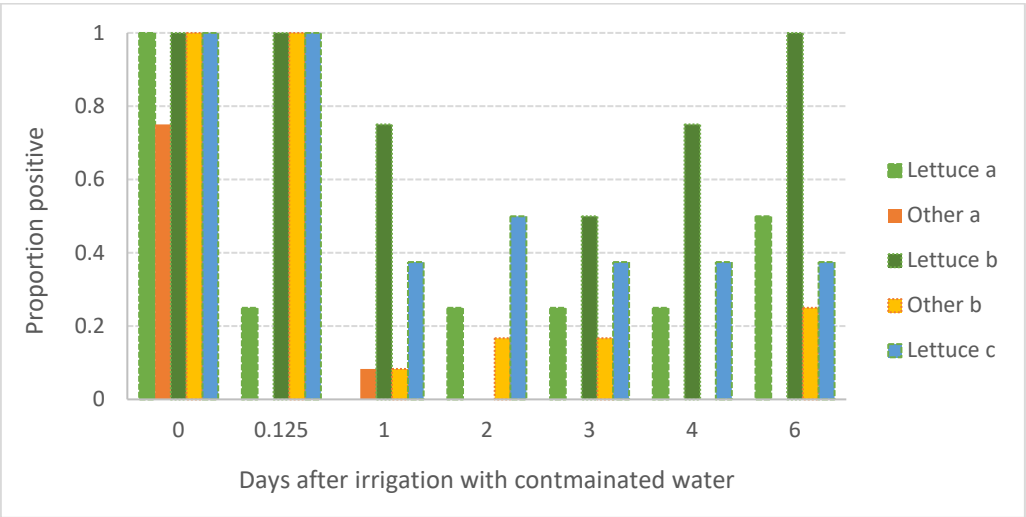

Figure 10. Populations of *E. coli* recorded on lettuces (n=4,8) and on parsley, chard and kale plants (n=12) after irrigation with contaminated water in three separate trials (a to c). Bars indicate the std. error of each mean value.

A high rate of detections of *Salmonella* spp., and occasional detections of *E. coli*, continued well after the recommended 48 hour withholding period, particularly for lettuce. It seemed likely that damage to the plants during transfer from the greenhouse to the open treatment area was allowing bacteria to persist on the leaf surfaces. Injury was most likely on the cos lettuce, their structure making them particularly easily damaged.

Trial 2 – Persistence of microbes from irrigation water on damaged and undamaged vegetables in the greenhouse

Gently compressing the leaves of the cos lettuce resulted in tiny cracks on the leaves, visible as leaking sap (Figure 11). The same level of damage was inflicted on chard and parsley, but injury was less obvious on the plants.

Figure 11. Lettuces, chard and parsley were either left untouched (left) or lightly damaged by compressing the leaves (centre), resulting in tiny cracks in the leaves (right)

For both A and B trials, *E. coli* populations, the first diluent solutions were tested and found to be close to the expected log 7 CFU/ml. Despite addition of diluent to the irrigation water, *E. coli* was below detectable levels in all samples tested. It is not known why this rapid die off occurred. As a result, *E. coli* was not detected on any plants in A, and only four damaged plants in B when tested immediately after irrigation.

However, *Salmonella* spp. was detected in both trials. There were nearly twice as many total detections on damaged lettuce compared to chard or parsley. There were no detections on intact vegetables when tested more than 24 hours after contamination, and no detections on damaged parsley or chard when tested three days or more after contamination. Only damaged lettuce continued to test positive for the full 6 days of the trial.

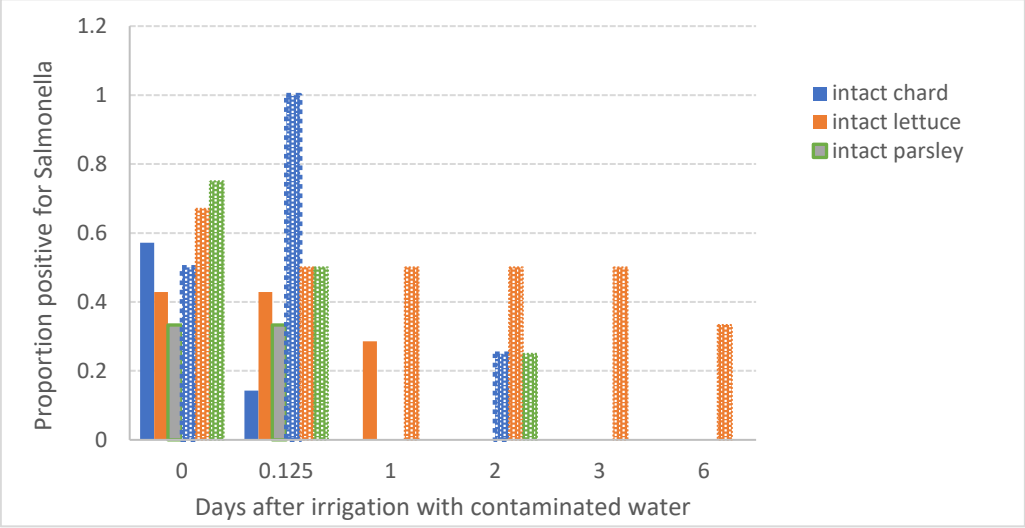

Figure 12. The proportion of tests that were positive for *Salmonella* spp for intact and damaged chard (n=7,4), lettuces (n=7,6) and parsley (n=6,4) after irrigation with contaminated water. Data combined from A and B trials.

These results demonstrated that damage increases persistence of pathogens on leafy vegetables, particularly lettuce, even if the injury is minor. A 48 hour withholding period between irrigation and harvest is not long enough to ensure products are safe if the water is contaminated and plants are damaged.

However, the results are supportive of the current 48 hour withholding period for undamaged product. This time period was sufficient for pathogens on the leaf surface to fall below detectable levels. This occurred despite relatively mild temperatures (20-25°C), high RH inside the experimental chamber and virtual

elimination of UV radiation by the greenhouse roof material.

Trial 3 – Persistence of microbes from irrigation water on damaged and undamaged vegetables in the field

To avoid the rapid die-off of *E. coli* that occurred in Trial 2, a mixture of three different non-toxigenic strains was supplied by Symbio laboratory. These also proved variable; mean populations of *E. coli* in irrigation water in trial A and B were log 3.62 CFU/ml and log 1.24 CFU/ml respectively. The trials therefore effectively tested differences in persistence when applied at high and low population densities.

Changes in *E. coli* populations were similar on undamaged lettuce, parsley and spinach plants, so this data could be combined for each trial (n=12/time).

There were few detections of *E. coli* in trial B, even when lettuces were damaged. In trial A, populations of *E. coli* on all intact plants were initially high, but declined rapidly. There were only two detections on plants sampled more than 3 hours after contamination. In contrast, detections on the damaged lettuces during trial A continued for six days. One sample recorded 7,000 CFU/g three days after irrigation. This suggests that damaging the plants not only allowed the bacteria to persist, but for the bacteria to multiply.

A much higher population of *Salmonella* was applied to the plants than would be expected to occur normally in irrigation water. Despite this, there was only one detection of *Salmonella* spp on undamaged vegetables 48 hours after the plants were irrigated; a single detection on spinach (A). In contrast, two of eight damaged lettuces tested six days after contamination remained positive for *Salmonella* spp.

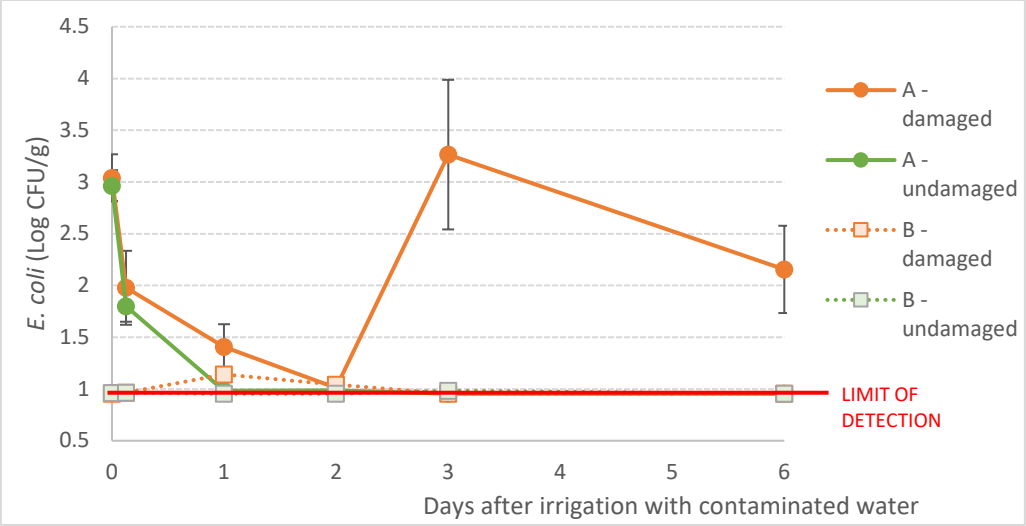

Figure 13. Changes in populations of *E. coli*, following irrigation with contaminated water containing high (A) or low (B) population densities of this bacteria, on undamaged lettuce, parsley and spinach plants (n=12) and damaged lettuces (n=4). Bars indicate the standard error of each mean value.

The results confirm that the risk of contamination is strongly affected by dose applied. Essentially, low levels of contamination pose less risk than high levels of contamination. This is consistent with other studies, which have found that bacteria are far more likely to persist and multiply when introduced at a high population density[xii, xiii].

There is therefore a strong risk that bacteria will persist and, potentially, increase if high populations of *E. coli* contact damaged plants. This is supported by previous reports of *E. coli* internalising and multiplying inside damaged lettuce leaves. For example, Brandl[xiv] reported that populations of *E. coli* O157:H7 increased 4x, 4.5x and 11x respectively on lettuce leaves that were bruised, cut into large pieces or shredded.

Trial 4 – Persistence of microbes on cos lettuce and spinach injured up to four days before irrigation with contaminated water.

Blocks of baby spinach and cos lettuces were initially damaged 72, 48, 24 or 1 hour before irrigation with contaminated water. After positive test results for the 72 hour treatment in A and B, this was increased to 96 hours for C and D. Damage was inflicted by trimming the tips of baby spinach leaves and the heart leaves of cos lettuce. In trials A and B, baby spinach was still small and some leaf yellowing was evident. As a result, tested samples included some material that would not have been commercial quality.

Figure 14. Damage to spinach and cos lettuce. Note the presence of yellow leaves on the spinach

Nearly all spinach plants were positive for *Salmonella* when sampled immediately after irrigation with contaminated water. A number of undamaged samples, as well as spinach damaged 24 hours or more before contamination, were still positive when tested two days later. However, this only occurred in trial A and, to a lesser extent, trial B. None of these samples were positive in trials C and D.

In contrast, wounding plants immediately before contamination resulted in detections across all four trials. It seems possible that the yellowed leaves included in samples from trials A and B had increased susceptibility to persistence by *Salmonella* spp.. Alam *et al* (2014) previously showed that infection with soft rots can increase persistence of *E. coli*[xiv]. Yellowing and/or slight downy mildew infection may also effectively be a type injury.

The opposite occurred for lettuces. Lettuces damaged 96 hours before contamination remained positive for *Salmonella* two days later. Even some nominally undamaged lettuces were positive from trials C and D. The lettuces used in the latter trials had been attacked by ducks a month prior to the trial; it seems possible that even this long delay still allowed increased persistence of pathogens from irrigation water.

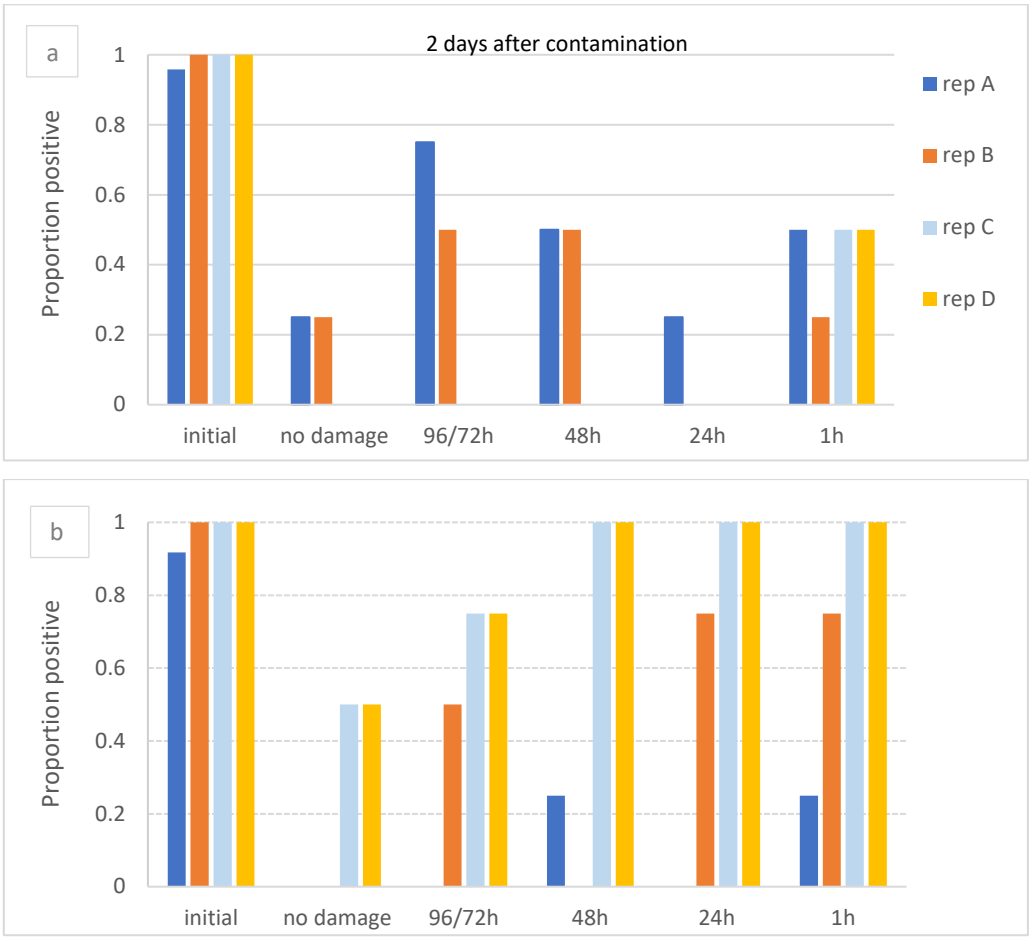

Figure 15. Proportion of spinach (top) and lettuces (below) from each of four trials that were positive for *Salmonella* initially and two days after irrigation with contaminated water. Plants remained undamaged or were damaged up to 96 hours before contamination.

Initial populations of E. coli were high on all plants. After two days, *E. coli* populations on spinach plants fell by an average of log 1.9 CFU/g (±0.08). While there was a trend to increased frequency of detections of *E. coli* when plants were damaged immediately before contamination, differences due to injury time were not significant.

In contrast to spinach, damage to lettuces increased the frequency of *E. coli* detection and resulted in significantly higher populations ($p<0.05$). Even damage that occurred four days prior to application of the contaminated water significantly increased the populations of *E. coli* that persisted two days later. The greatest effect was found when damage occurred immediately before contamination; after two days, populations of *E. coli* on these lettuces were significantly higher than all other treatments ($p<0.05$).

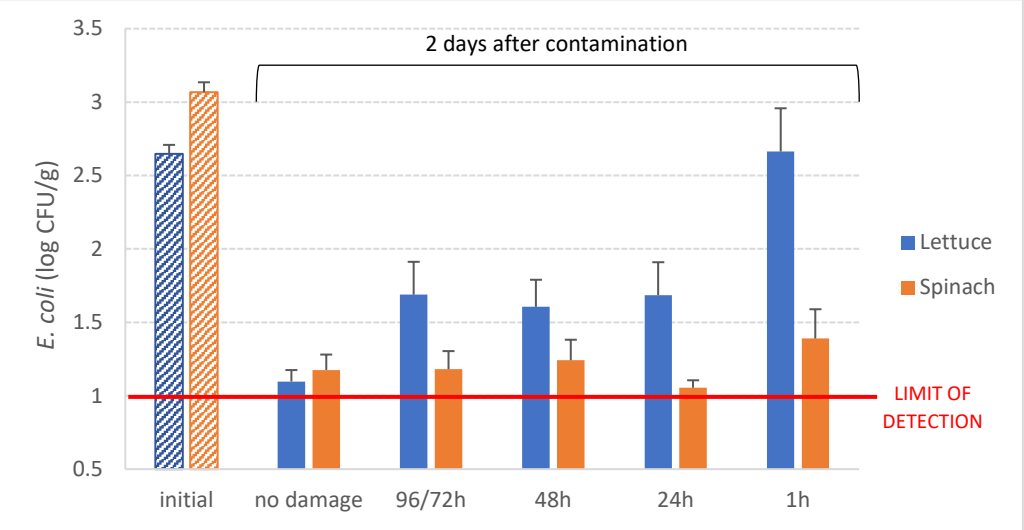

Figure 16. Mean log populations of *E. coli* on spinach and lettuce plants that remained undamaged, or were damaged up to four days (96h) before irrigation with contaminated water. Bars indicate the standard error for each mean value (n=12).

The results suggest that *E. coli* is no more likely to persist on spinach damaged 24 hours or more before irrigation than on undamaged plants. However, no "safe" period was identified for cos lettuce. Damage 96 hours, and possibly longer, before contamination increased persistence of both *E. coli* and *Salmonella*. Damage immediately before irrigation had the greatest effect. It is concluded that great care should be taken to avoid irrigating cos lettuce with contaminated water at any time.

Detailed results in Appendix 4.

Monitoring and evaluation

The project achieved the intended outcome to examine further in an Australian context some of the recommendations in the Guidelines for Fresh Produce Food Safety. Those included withholding periods between application of untreated or semi-treated manures and harvest of vegetables, and guidelines for the safe use of pre-harvest water on vegetables.

That was achieved in part by guidance from a diverse project team, and project reference group. Regular meetings (Table 6) with those groups meant the procedures used and results gained were industry relevant and met the needs of the whole supply chain.

Each meeting included an update on research progress and discussion of next steps. Progress was logged against the expected project timeline (Figure 17). This changed as the project progressed. For example, Task 5b – "Pathogen survival in soil field conditions" was intended to examine die off rates of human pathogens after addition of manures to soil on commercial vegetable farms. However, pathogens were either not detected, or at already very low levels, in samples of manures on farms. Moreover, commercial sensitivities about this issue made this approach difficult. This activity would likely not have provided any useful information regarding die off rates or risk associated with manures. The activity was therefore re-designed, resulting in the series of semi-controlled trials conducted at Cobbitty.

A second example is the research on irrigation water. The initial results from irrigation trials (Trial 1a-c) were discussed with the reference group. Dr Robert Premier was able to then present the results of some of his previous research with Dean Harapas on the effect of shoot damage on persistence of pathogens on vegetables. This guided the following activities, which examined this issue in more detail.

Table 6. Dates of project team and reference group meetings

Date	Group
3/11/17	Initial project team and reference group meeting
7/12/17	Project team
16/2/18	Project team
4/4/18	Project team
28/6/18	Project team
12/9/18	Project reference group
20/12/18	Project team on site at Cobbitty
18/7/19	Project reference group

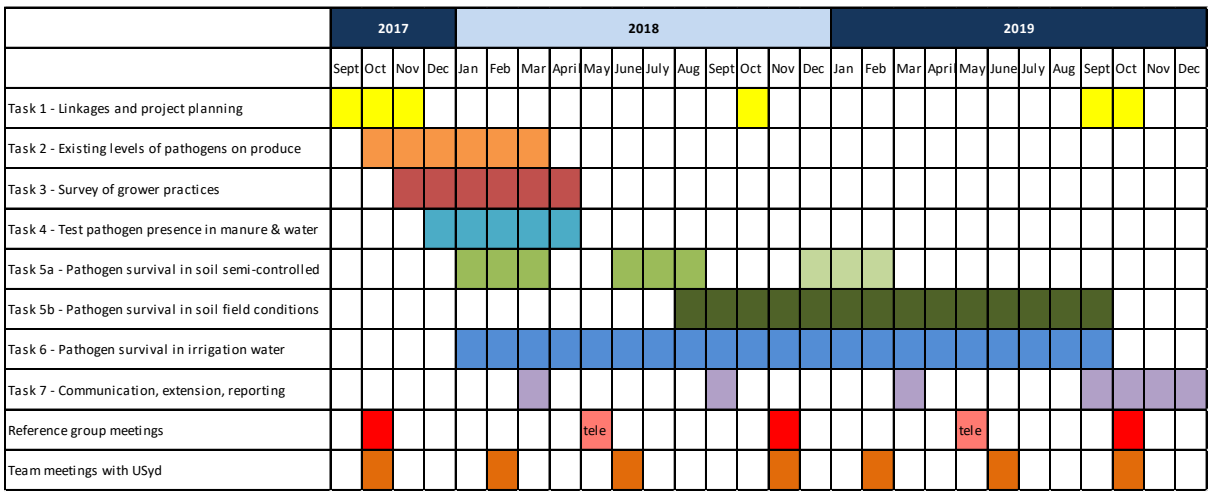

Figure 17. Project timeline GANNT chart

The reference group members included:

Brendan Hayes – Coles
Brice Lamarque – Woolworths
Andrew Shaw – AUSVEG
Belinda Millard – HARPS
Prof. Robert Barlow – CSIRO
Andrew Francey – OneHarvest
Thea King – NSW Food Authority

The wider project team included:

Dr Jenny Ekman – Applied Horticultural Research
Adam Goldwater – Applied Horticultural Research
Prof. Robyn McConchie – University of Sydney
Dr Mark Bradbury – University of Sydney
Dr Hannah Sassi – University of Sydney
Dr Tina Bell – University of Sydney
Clare Hamilton-Bate – Freshcare
Jacinta Fong – Freshcare
Dr Robert Premier – GSF/Consulting environmental microbiologist
Richard Bennett – Fresh Produce Safety Centre / PMA Australia

Having such a large group of people involved and overseeing project activities during the project guided project activities and ensured that the project remained on-track to meeting objectives at all times.

Recommendations

Produce sampling

The results indicated that contamination of vegetables is very low, with less than 1% of samples testing positive for any human pathogen. However, detections were higher on leafy salad greens, particularly those grown in southern regions.

It would be extremely valuable to re-visit this data regularly. This would verify that food safety standards are being maintained or improved, providing a benchmark for changes over time. While not expensive (as the data already exists), such an activity could provide early warning of food safety issues, which may increase or decrease as changes in temperature and water availability impact vegetable farmers.

This could potentially go across all industries, as this is a common issue for all of horticulture.

Background levels of pathogens in manure

Research has identified that individual cattle in feedlots, can be "super-shedders" for human pathogenic *E. coli* (>log 4 CFU/g feces). The number of cattle affected ranges from 2.8% over 20% of the herd[xv]. Concentrated animal feeding operations (CAFOs) such as feedlots are an important potential source of contamination to horticulture. The 2018 outbreak of *Listeria monocytogenes* associated with rockmelons has been connected with wind-borne dust generated by a CAFO several kilometers away[xvi]. The 2018 outbreak of *E. coli* O157:H7 on cos lettuce in the USA has also been traced back to a CAFO, in this case a feedlot located near an irrigation canal[xvii].

According to the Australian Lot Feeders Association, strong demand for export cattle (and sheep) has been driving rapid expansion in feedlots. The extent to which this creates new risks for horticulture is poorly understood. A desktop review of research on this issue would provide guidance on likely risk, while testing of waste products is essential to prevent accidental contamination of horticultural products.

The Australian Meat and Livestock Association recently commissioned new research into the types and quality of waste product produced by livestock industries. Investigating the suitability of these products for horticulture would make a valuable cross-sector research study with excellent opportunities for data sharing.

Die off rates of pathogens in manure amended soils

The results of the trials conducted in this project are generally supportive of the current withholding periods between application of manures and harvest. However, microbial data is highly variable, with these trials representing a limited snapshot of changes in microbial populations in soils. Most overseas studies of die off rates have been conducted over several years rather than, as this study, over a single 10-month period. These have found significant annual differences due to climate, soil moisture, incorporation method and other factors.

Repeating these trials over time would greatly improve the robustness of this data. In particular, trials should be conducted in a more southern region, these having been identified as the highest risk based on the survey of microbial quality of produce.

The University of Sydney is collaborating with the Western Centre for Food Safety at UC Davis, USA on a QMRA for water quality and have been invited to join an FDA trial looking at pathogen persistence in manure amended soils in a wide range of geographical and environmental settings. Trials would use a standard protocol to ensure valid comparisons. The data from Australia would be an important part of this data set and assist in clarifying persistence ranges in Australia.

Further, harmonisation of future study methodologies and target pathogen reductions can provide support to ensure that Australian guidelines remain consistent with global best practice and support market access for Australian produce internationally.

Reducing risk from irrigation water

In general, water presents a greater food safety risk than manure[xiii]. Most food safety outbreaks involve

contaminated water. The risk from water is highlighted by our results demonstrating increased persistence and multiplication of pathogens on damaged vegetables. It seems possible that most food safety outbreaks also involve injury – whether by storm damage, disease, pests or other factors. In particular, harvesting leafy greens while wet may create a very high level of risk, as human pathogens can readily internalize in the cut surfaces.

The results were suggestive, although definitely not conclusive, that even damage 4 weeks before contamination by irrigation water increased persistence of *Salmonella* on cos lettuce. They were also suggestive that low levels of downy mildew infection and/or leaf yellowing increased persistence on spinach.

It is strongly recommended to continue research on this important issue, examining;

- The effect of time interval between damage and contamination for a range of leafy salad greens, including time intervals of up to 4 weeks
- The effect of mild fungal infection (below commercial thresholds) on persistence of human pathogens on vegetables, including root crops (e.g. carrots) growing in manure amended soil
- Determine whether physical damage due to strong wind or rain, insect feeding, or movement of farm equipment (e.g. multiple harvesting) increase risk
- Further investigate critical limits for irrigation water, given that high levels of human pathogens in water can persist and internalize, whereas this likelihood appears far less with low initial populations
- Review whether toxins in irrigation water contaminated with blue-green algae transfer to plants, and the time required for these products to be de-toxified on damaged/undamaged plants
- Test in-line solutions to reduce human pathogens in irrigation water e.g. solar-powered UV or e-water systems for field application.

Refereed scientific publications

None as yet, two papers currently at DRAFT stage.

Intellectual property, commercialisation and confidentiality

None to report

Acknowledgements

This project represents the combined efforts of a large group of researchers, field staff, laboratory staff, technical advisors and others. These include:

Dr Jenny Ekman, Adam Goldwater and Dr Gordon Rogers – Applied Horticultural Research

Richard Bennett – Fresh Produce Safety Centre / PMA Australia

Prof. Robyn McConchie, Dr Mark Bradbury, Dr Hannah Sassi, Ms Emily White and Katarzyna Safianowicz – University of Sydney, SOLES

Glen Foxwell, Paul Lipscombe and James Bell – University of Sydney, Cobbitty

Ben Thew, Sarah Kelly and Imtiaz Ahmed – Symbio Laboratory

Clare Hamilton-Bate, Fiona Grime and Angela Steain – Freshcare

Dr Robert Premier – GSF/Consulting environmental microbiologist

Jessica Purbrick and Emma Walters – Fresh Produce Safety Centre Australia & New Zealand

Prof. Robert Barlow – CSIRO

Brice Lamarque – Woolworths

Brendan Hayes – Coles

Andrew Shaw – AUSVEG

The project also acknowledges the input of vegetable growers who provided information on their use of soil amendments and samples of products used on-farm.

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