Reducing Listeria contamination from salad vegetable farms

Robert Premier
Global FS Pty Ltd

Project Number: VG07079

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This report is published by Horticulture Australia Ltd to pass on information concerning horticultural research and development undertaken for the vegetables industry.

The research contained in this report was funded by Horticulture Australia Ltd with the financial support of the vegetables industry.

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ISBN 0 7341 2463 5

Published and distributed by:
Horticulture Australia Ltd
Level 7
179 Elizabeth Street
Sydney NSW 2000
Telephone: (02) 8295 2300
Fax: (02) 8295 2399

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Final report for HAL project

(July 2010)

Robert Premier

Global F.S. Pty Ltd

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Final report for HAL project VG07079

By: Robert Premier

Project Leader
Dr Robert Premier
Global F.S. pty ltd
Ph: 0418317786
Email: robert.premier@consultant.com

Scope of the Report
This report presents the key findings and a summary of the work conducted in Victoria and Queensland from September 2008 to June 2010 by the Project team.

Funded By:
Horticulture Australia Limited and the Australian vegetable industry levy.

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Acknowledgements:

The project team would like to acknowledge the support and input from all those that have contributed to the project including the participating growers that wish to remain anonymous. In addition the team would like to acknowledge Dean Harapas, Dr Said Ajlouni from the School of Land and Environment, University of Melbourne, Sandy Clarke from the University of Melbourne statistical consulting centre and Dr Marie-Astrid Ottenhof for reviewing the manuscript.

1. Media Summary

Listeria monocytogenes (*L.monocytogenes*) is a bacterium that has been widely detected in the environment and plants, including leafy vegetables. It has the potential to become a human pathogen, affecting young people, older people and pregnant women. Every year in Australia a number of people die as a result of *L. monocytogenes* infections. Supermarkets displaying due diligence have set very tight specifications that vegetable growers must comply with. This, in turn, has led to growers having to pre-test their products for the presence or absence of *L. monocytogenes* as a condition of supply to supermarkets. The problem, however, is that with *L. monocytogenes* testing, there are a number of tests available and often the grower is confused as to which test should be used. More importantly the major problem is that growers have no idea how to reduce the incidence of *L. monocytogenes* in the field and they have no idea how this bacterium enters farming land to contaminate their crops. What is needed is an information package on this issue for the grower.

This project had three aims. The first aim was to understand how *L. monocytogenes* enters a vegetable farm from the environment. The second aim was to understand if a rapid detection method can be of assistance to the grower and to make recommendations on what method the grower should be asking for when getting produce tested by laboratories. The third aim of this project was to produce an information sheet on testing and on ways to reduce *L. monocytogenes* issues on farm.

In order to understand how *L. monocytogenes* enters the farm, a number of samples were taken from three vegetable growing sites in Victoria and two vegetable growing sites in Queensland. Samples of produce, soil, water and manures found on farms were taken and tested for the presence or absence of *L. monocytogenes*, in most cases when positives were detected the numbers of *L. monocytogenes* were quantified.

The results of this project suggest a possible pathway for how *L. monocytogenes* contamination occurs in vegetable farms. *L. monocytogenes* was found to be present in high numbers in silage and fermented baled hay, which are both, in turn fed to and ingested by ruminants; this passes through the animals usually without causing infection to them. The *L. monocytogenes* remains trapped within the matrix of the faeces and disperse only when the faeces becomes dry in hot weather (a process assisted by wind and heat). The dust carrying the *L. monocytogenes* is then spread by strong winds covering large distances, where it can contaminate vegetables through the dust particles. Leafy vegetables (e.g. curly parsley) that can trap dust more effectively usually show higher levels of detection than smooth leaf vegetables, such as cos lettuce. The results of this study clearly show that after windy and hot days the level of detection on leafy vegetables can potentially increase.

Testing for *L. monocytogenes* by growers should be based on the Australian standards and any *L. monocytogenes* detected must be enumerated. Information based on the findings of this project and recommendations for reducing the incidence of *L. monocytogenes* on produce before delivery to markets are discussed in easy to understand terms.

More work needs to be carried out to determine the virulence of the subtypes of *L. monocytogenes* found in vegetables, as these are more than likely to be of low virulence as their origin is from plant matter and not infected animals.

2. Technical Summary

The aim of this project was three fold. The most important aim was to get a better understanding of how *L. monocytogenes* enters vegetable farms. This information is important if vegetable growers are to minimise risks at on farm level. The other two aims of this project were to understand what is the best type of test that growers need to specify for the testing of produce and to develop information for the grower to use when dealing with *L. monocytogenes.* This project is designed to gather information to aid in the management of food safety in leafy vegetable farms in Australia.

L. mocytogenes is one of the bacteria that must be tested for to gain market entry with supermarkets and fresh cut processors in Australia. *L. monocytogenes* has been detected widely in Australian leafy vegetables in the past and rejections of produce associated with its detection have occurred on numerous occasions. Herb and vegetable growers have lost income as a result of these rejections or supply being placed on hold by customers until testing shows that all is clear.

This project

- examined how *L. monocytogenes* is tested for and looked at the value of a rapid testing method
- carried out testing on produce both in Victoria and Queensland in summer and in winter over a period of two years
- carried out testing on environmental samples including water, sheep manure, cow manure, chicken manure, marsupial manure and decomposing plant matter to try and identify reservoirs of *L. monocytogenes* in the environment
- produced a guide for vegetable and herb growers to use when dealing with *L. monocytogenes* at on farm level
- extended the knowledge of *L. monocytogenes* so that more work can now be done to decrease this problem for growers
- extended information to industry on practices to better manage *L. monocytogenes* contamination issues

3. Key Outcomes and Conclusions

- Australian standard method based on culture and enumeration is the preferred method to use when testing for *L. monocytogenes.*
- Rapid testing is not the preferred manner to detect *L. monocytogenes* on produce but can be used as a preliminary screening test followed by culture and enumeration
- *L. monocytogenes* is more prevalent in summer; this is the opposite of what is reported in overseas literature, applying to the colder climates in the Northern hemisphere
- Wind appears to be one of the vectors that spreads *L. monocytogenes*

- *L. monocytogenes* appears to originate in ruminant faeces, an observation supported by international literature

- The animals, however, are not sick and the *L. monocytogenes* appears to originate from animal feed that is fed to ruminants during summer

- *L. monocytogenes* is not predominantly spread by water and chicken manure, two common farm inputs in Australian vegetable farming

Recommendations for Future Work

- A better understanding of the virulence of windborne *L. monocytogenes* originating from decomposing plant matter

- The development of a test that targets virulence genes may shed new light on the issue of *L. monocytogenes* for vegetable and herb growers

- More testing in other areas of mainland Australia and Tasmania may also shed new information on how widespread this problem is for growers

4. Introduction

L. monocytogenes is a human pathogen that can potentially cause serious disease outbreaks in humans. The Australian Food safety guidelines for fresh produce specify 100 CFU (colony forming units) of *L. monocytogenes* per gram as being the upper limit allowed in leafy vegetables. The level accepted however is dictated by the customer and *L. monocytogenes* is one of the bacterial pathogens that is usually specified in approved supplier contracts. In Australia, the major supermarkets require *L. monocytogenes* to be absent or present in very low levels (less than 10 CFU/g) in fruit and vegetables. It is also required to be absent or present in very low levels in salad vegetables that are supplied to minimally processed vegetable (fresh cut) manufacturers. There are many aspects of *L. monocytogenes* that are not understood, for example, we do not know why at times it is so prevalent in Australia in leafy vegetables, we do not know the vectors that are spreading this human pathogen in farming areas and we have no idea for how long it survives in Australian situations. More importantly, there are views that water may be responsible for the spread of *L. monocytogenes* but with the scarcity of this resource, there is no possibility for a grower to easily change water supplies, should this be the case.

The aim of this project is to answer some of these questions, the results of which will be used to write a best management practice guide to reduce the incidence of *L. monocytogenes* contamination in the Australian vegetable industry.

L. monocytogenes is a gram positive to gram variable aerobic and microaerophilic non spore forming bacterium. Even though it does not have the protection of the spore, the bacterium is very hardy and survives well in a range of environments. It is widespread in soils throughout the world, however, it can also be found in a large number of animals and birds including cattle, sheep and poultry (Beuchat 1996). It causes the disease listeriosis, symptoms of which can be severe and include bacterial meningitis, endocarditis (inflammation of the membrane lining of the heart) and peritonitis (inflammation of the membrane lining the cavity of the

abdomen). Perinatal infections can also occur resulting in abortion or stillbirth. It can affect all age groups but can be fatal in neonates and foetuses leading to extensive warnings for pregnant women as to the danger of this disease. It is also a major problem in immunosuppressed individuals.

The prevalence of listeriosis has been increasing globally, the result of an increase in the rate of predisposing factors in the community. These predisposing factors include neoplastic disease, drug induced immunosuppression, alcohol abuse, diabetes mellitus, cardio-renal diseases, old age and disease induced immunosuppression (AIDS).

Foods have been established as a major vector for the spread of listeriosis and it is associated with a mortality rate of 30% in food outbreak situations. A number of overseas studies have examined various fresh vegetables for the presence of *L. monocytogenes.* Sizmur and Walker (1988) looked at 60 samples of prepacked salads purchased from two leading supermarkets in the UK. Four samples, representing 2 salad varieties, were found to contain *L. monocytogenes.* One of the salads contained cabbage, celery, sultanas, onion and carrots whilst the other one consisted of lettuce, cucumber, radish, fennel, watercress and leeks. Heisick *et al.* (1989) conducted 1000 tests on 10 types of fresh produce from two supermarkets in the Minneapolis area in the USA. *L. monocytogenes* was isolated from cabbage, potatoes, cucumbers and radishes, although only potatoes and radishes contained significant amounts. Other studies have isolated *L. monocytogenes* from bean sprouts, leafy vegetables, prepacked salads, salad vegetables and tomatoes in Malaysia, the USA, Germany, Ireland and Pakistan (Beuchat 1996) and leeks, potatoes, lettuce, celery and cabbage in Spain (de Simon *et al.* 1992). A study of food in Taiwan detected *L. monocytogenes* in 12% of vegetable samples tested (Wong *et al.* 1990). *L. monocytogenes* has also been isolated from frozen products including vegetable soup, asparagus and cultivated mushrooms (Gola *et al.* 1990). A study was conducted in NSW during 1988 to 1993 on different types of foods, including 54 ready to eat vegetable and salad samples (Arnold and Coble 1995). Only 1 sample was found to be positive for *L. monocytogenes.* Recent studies in Malaysia have found that out of 306 vegetables that are consumed in a ready to eat form, 22.5% had detectable levels of *L. monocytogenes* (Jeyaletchumi Ponniah *et al.* 2010), in Chile 10.2% of supermarket samples had detectable levels of *L.monocytogenes* (Cordano and Jacquet 2009).

There have been outbreaks attributed to the consumption of fresh produce overseas. During the summer of 1979, 23 patients admitted to hospital in Boston had systemic *L. monocytogenes* infection (Ho *et al.* 1986). It was concluded that raw celery, tomatoes and lettuce contaminated with *L. monocytogenes* may have been responsible. An epidemic in 1981 involving both adult (7 cases) and perinatal (34 cases) infection, was linked to raw cabbage in coleslaw (Schlech *et al.* 1983). An investigation into the sources of the cabbage found that one farmer had used composted and fresh sheep manure. Two of his sheep had died of listeriosis in 1979 and 1981.

Crerar *et al*. (1996) reported two outbreaks which occurred in Australia, affecting 13 people, one of which was linked to seafood and in the other outbreak the food vehicle was unknown. *L. monocytogenes* from horticultural foods has been held responsible for a number of deaths in Australia, the most notable of which were the deaths of 6 nursing home residents in NSW in 1999 after the consumption of a fruit salad (Australian Associated Press, 1999).

This has led to a greater scrutiny of fresh produce as a source of *L. monocytogenes* contamination, particularly fresh produce that is grown close to the ground and is eaten

uncooked. Most State Departments of Health require mandatory reporting if *L. monocytogenes* is detected in food. When it is detected, the contamination is investigated making it difficult, if not impossible, for producers to sell their produce until all is cleared by the Department of Health. What is becoming clear is that there are sporadic high level contamination events in the fresh produce industry and we do not understand how these occur.

Overseas literature cannot be extrapolated to the Australian situation with ease because *L. monocytogenes* seems to be more of an issue in Australian farming systems, especially the fresh salad vegetable sector. Every so often growers of leafy vegetables, particularly curly parsley, are notified by supermarkets to cease supply as *L. monocytogenes* was detected in their products. More importantly, every year producers of fresh cuts (minimally processed vegetables) live with the threat of product recalls due to the detection of *L. monocytogenes* in their products. This is often traced back to the farm and the recall affects the profitability of the grower. Recalls also give the overall vegetable industry a bad reputation with customers and consumers, reducing purchases of these products for some time after a publicised general product recall. Testing for *L. monocytogenes* in vegetables can take as long as 7 days, however new rapid tests may be adapted and can potentially reduce this time frame to 48 hours or less.

Rapid detection could assist industry in establishing if the problem has cleared up faster than conventional testing. This project will seek to understand how *L. monocytogenes* enters the growing chain, what vectors are involved and how long *L. monocytogenes* survives for in Australian farming systems. It will seek to develop or adapt an existing rapid *L. monocytogenes* test for use in the vegetable industry and to make recommendations to growers as to what is the best test to use to detect *L. monocytogenes* in their produce. This information combined will be used to develop a best practice tool to reduce *L. monocytogenes* contamination of vegetable farms in Australia.

The project is designed to gain a better understanding of where *L. monocytogenes* is found on farm that produce leafy vegetables in Australia, in particular the project aims at finding out how this human pathogen enters farms and contaminates leafy vegetables. The object of this study is to develop practices that will reduce the incidence of *L. monocytogenes* on leafy vegetable farms in Australia.

5. Evaluation of rapid tests for the detection of *L. monocytogenes* in fresh produce.

5.1 Introduction:

L. monocytogenes can be detected in a number of ways, in the horticultural industry the grower often has no idea what test to specify, usually the grower allows the laboratory to use a rapid test procedure that is of their choosing. A variety of culture and rapid methods are available for the detection of *Listeria* species and *L. monocytogenes* in foods.

There are 6 species of *Listeria* that are found in the environment, only one is pathogenic to humans. *Listeria innocua, Listeria welshimeri, Listeria seeligeri, Listeria grayi, Listeria*

ivanovii are considered to be non pathogenic to humans (pathogenicity of *Listeria ivanovii* is still debated) whereas *L. monocytogenes* has been implicated in human and animal disease. The presence of other species is often used as a surrogate marker of the probable presence of *L. monocytogenes*. Growers should, however, always specify a full *L. monocytogenes* test as opposed to a genus test (testing for *Listeria* species in general). This is because there is a much higher probability that on leafy vegetables grown in the field there may be contamination from a non pathogenic *Listeria* species, thus the detection of a *Listeria* species other than *L. monocytogenes* would be detrimental to the grower when it comes to reporting the test result. Therefore, the most adequate tests are those which specifically detect *L. monocytogenes*. Additionally, supermarkets specify *L. monocytogenes* testing and not genus testing. Table 1 shows the microbiological supermarket requirements, including requirements for *L. monocytogenes* in both fresh produce and minimally processed vegetables.

Table 1: Supermarkets *L. monocytogenes* specifications and Australian guidelines for produce and fresh cuts.

Coles supermarkets	Ready to eat leafy vegetables Absent in 25 g	Fresh produce Not detected in 25g
Woolworths Supermarkets	Ready to eat leafy vegetables Less than 10 CFU/g	Not mandated but where risks have been identified less than 10 CFU/g
Australian guidelines	Ready to eat Less than 100 CFU/g	Fresh produce food safety guidelines Less than 100 CFU/g

In the absence of instruction from growers, rapid tests are often used and the performance of these tests is usually not discussed when presenting results. Two widely used testing procedures are chromogenic culture tests and enzyme immunoassay tests.

There is a third class of rapid testing available but is not used widely, this is a PCR based DNA amplification test. PCR testing has been around for a long time. Some PCR tests are available commercially for *L. monocytogenes* but these have protocols specific to one application; no application has been found specific for the detection of *L. monocytogenes* in horticultural products, most likely because of the difficulty of bacterial extraction and to the selective enrichment steps that must incorporated to recover *L. monocytogenes* nucleic acid that is suitable for testing. There is the belief that a PCR based test for *L. monocytogenes* isolated from leafy vegetables would save a lot of time and could potentially assist with making firm decisions as to rejection of product and cessation of purchasing from growers by supermarkets or fresh cut producers. This belief however has never been proven, as no DNA based tests for *L. monocytogenes* in fresh produce are available commercially. This component of the project aims to develop such a test and to compare it to the current methods for the detection of *L. monocytogenes* in leafy vegetables. It also aims to answer the question often asked, can a rapid method for the detection of *L. monocytogenes* assist leafy vegetable growers.

This section of the project utilised a published PCR test for *L. monocytogenes* and adapted it for the testing of leafy vegetables. An assessment was then made as to how useful this type of test would be for vegetable growers

5.2 Material and methods:

Test development: A published PCR test for *L. monocytogenes* was used as a template to develop a test specific for use in the leafy vegetable industry. VIDAS (LMO2) test kits were used as described by the manufacturer. The culture tests were carried out as described in the Australian standards (Australian Standard AS 1766.2.16.1-1998: Food microbiology - Examination for specific organisms - Food and animal feeding stuffs - Horizontal method for the detection and enumeration of *Listeria monocytogenes*).

Sample enrichment and culture: Samples were transferred to sterile stomacher bags and homogenised directly in enrichment medium at medium speed for 5 minutes. The homogenised material was incubated at 30º C for 48 hours before testing in culture, VIDAS and PCR. For hay samples and manure samples the enrichment step was followed by a selective step and another enrichment step at lower temperatures (8º C for 24 hours). This assisted with heavily contaminated samples and selected out *Listeria* better than conventional enrichment alone.

***L. monocytogenes* strains and samples used to evaluate the test**: All samples were isolated over a period of 3 years at DPI (Victoria) and the University of Melbourne and typed and tested by NATA registered commercial laboratories

A panel of sixty samples tested using the Australian standard method was used to evaluate the efficacy and specificity of the rapid tests. The panel consisted of 30 known positives for *L. monocytogenes*, 20 known positive for *Listeria* species (not including *L. monocytogenes*) and 10 negative for any *Listeria* species (5 *E.coli* isolates and 5 Salmonella spp. isolates).

PCR test: A rapid test was developed for the sole purpose of detecting *L. monocytogenes* in leafy vegetables. This test, based on PCR technology, was also compared to an existing rapid test (VIDAS) and the standard culture based method, as described in the Australian standards.

Hence two rapid tests were examined for their suitability for use in the horticultural industry. These included the VIDAS (Vitek Immuno Diagnostic Assay System) (LMO2) (Biomeriux, Australia) and a test based on PCR technology. These were compared to the Australian Standard method for *L. monocytogens* detection that can take up to 7 days to complete (Australian Standard AS 1766.2.16.1-1998: Food microbiology - Examination for specific organisms - Food and animal feeding stuffs - Horizontal method for the detection and enumeration of *Listeria monocytogenes*). VIDAS takes approximately 48 hours and the PCR test can take as little as 24 hours to complete but realistically, because of enrichment steps, a total time of 48 hours is required. Both rapid tests require an incubation period in enrichment media.

VIDAS testing and Culture: VIDAS testing was carried out in accordance with the manufacturer's recommendation; basically all samples were homogenised and enriched using Demi Fraser broth and incubated at 30 ºC for 24 hours. 0.1 ml of this was then transferred to

10 ml of Fraser broth without FeNH4Cit and incubated at 30 ºC for 24 hours. Two ml of this were then heated for 15 minutes at 95 ºC and tested with the VIDAS LIS kit. All positive samples went through a selective isolation and were confirmed as *L. monocytogenes* positive with the use of *Listeria* chromogenic agar by detecting Blue / Green colonies with halo zones.

Sample preparation for PCR testing: 25 g of vegetable matter was placed into stomacher bags (sterile) and homogenized with a stomacher in 225 ml of half concentrated Fraser broth (Oxoid) for 1 min. This was incubated at 30 ºC for 24 hours and was considered as the pre-enrichment step. An enrichment step then followed by using 100 µl of this material into 10 ml of Fraser broth and again incubated at 37ºC for 24 hours. After pre-enrichment and enrichment steps, 100 µl of the enrichment solution was diluted 1:10 in sterile distilled water and kept at -20 ºC for PCR analysis.

DNA extraction: Tubes containing tissue were centrifuged at 20,000 *g* for 5 minutes. After centrifugation, the supernatant was discarded and the remaining pellet was suspended in 200 ml of a commercially available reagent and boiled for 10 min. After boiling, tubes were centrifuged at 15,000 g for 5 minutes. Fifty microliters of supernatant from each sample containing extracted DNA were mixed with 50 ml of Tris-EDTA buffer and stored at 4 ºC. This final solution was used as the DNA template in the PCR reaction. The DNA extracted from pure bacterial cultures was quantified using a spectrophotometer at 260 nm and adjusted to 1 1µmmol^{-1}.

PCR primers and PCR conditions: The PCR procedure used was basically as described by P.A. Gouws and I.Liedermann (2005). Each reaction was performed in a volume of 50 µl with the following components: 1µmmol^{-1} of purified chromosomal DNA. Primers (Monash University) consisting of 5'-CATTAG TGG AAA GAT GGA ATG -3 and primer 5'-GTA TCC TCC AGA GTG ATC GA -3'were used in a reaction mixture of 100 µmmol^{-1} of each dNTP; 1.5 mmol / MgCl2; 5 ml 10r Taq buffer and 0.5 U Taq polymerase (Promega). The amplification reaction was carried out on a Hybaid thermocycler using the following conditions: first, the denaturation step at 94 ºC for 5 min., followed by 30 cycles consisting of: denaturation at 95 ºC for 30 s, annealing at 55 ºC for 30 s, extension at 72 ºC for 30 s. Final extension was carried out at 72 ºC for 2 min. Control samples were included in each PCR. The positive control was in-house standard containing approximately 100 *L. monocytogenes* cells ml. In the negative control, 1 µl of sterile distilled water was used instead of bacterial cell lysate.

The amplified DNA was analysed by gel electrophoresis on a 1% agarose gel stained with ethidium bromide. A 125 bp ladder (Promega) was used as a reference marker. the amplified segment of 730 Base pairs was visualised using a UV illuminator at a wavelength of 254 nm.

5.3 Results:

A panel of sixty samples tested using the Australian standard method was used to evaluate the efficacy and specificity of the rapid tests. The panel consisted of 30 known positives for *L. monocytogenes*, 20 known positive for *Listeria* species but not including *L. monocytogenes* and 10 negatives for any *Listeria* species.

As expected the standard method picked up all the positives for *L. monocytogenes,* it did not pick up any of the negatives for *L. monocytogenes.* The VIDAS picked up 29 of the positive samples but it also picked up three of the samples containing *Listeria* species but not *L.*

monocytogenes. It also picked up two of the negatives as a positive for *L. monocytogenes*. The PCR test detected all of the positives and four of the negatives (three of the *Listeria* species containing samples and one non *Listeria* negative) as a positive. Based on these results the PCR test had a specificity of 86.7% and a sensitivity of 100%, the VIDAS test had a specificity of 93.4% and a sensitivity of 96.7%. Where the sensitivity measures the proportion of actual positives which are correctly identified as such and the specificity measures the proportion of negatives which are correctly identified as negatives. These results do not differ widely from published data. It is important however to understand that the reproducibility of the PCR assay depended largely on the efficacy of the enrichment steps to work correctly. It is worth mentioning that the efficacy of the PCR is also directly related to the nucleic acid sequence targeted. The oligonucleotide primers that are chosen as amplification points are also very important. If these are not standardised, there is a risk that the sequences chosen are not present in all isolates of *L. monocytogenes.*

Both the VIDAS and the PCR test could be completed in 48 hours, as opposed to the Australian standards culture method, that required up to 7 days testing for comparable sensitivity and specificity.

6. Testing for *L. monocytogenes* on farms

6.1 Method:

Three Victorian vegetable growing areas have been targeted for the study. This includes areas around Melbourne (the Mornington peninsula), the Bacchus Marsh area and the lettuce growing area of Gippsland. A number of growers were contacted and gave their approval for project staff to sample on their property. Farms were coded so that no immediate identification of actual farms could be made. Farms targeted included baby leaf growers, herb growers and head lettuce growers.

Similarly three farms were selected in Queensland for inclusion in this project; all farms were in the Gatton area.

Testing took place over two years in both summer and winter. Summer testing took place during the months of January, February and March. Winter testing took place between the months of June to October for year 1 and in the months of June and July in year two. Samples of produce tested included equal numbers of cos lettuce, fancy lettuce (oak, coral or butter), curly parsley/coriander and, when available, celery.

A general sampling plan was used and samples were taken in a zig zag pattern from each farm, this included soil samples and produce samples. This ensured a representative sample across the whole growing area. Produce samples were taken using gloves and placed immediately into a sterile bag and stored in an esky with ice before being taken back to the laboratory for testing. Soil was taken from the surface of the growing area and placed in a sterile plastic container before being transported in ice for storage at 4 ºC in the laboratory. Manure samples were placed in sterile bags and transported in ice for storage at 4 ºC until

tested in the laboratory. Water samples were collected in sterile 250 ml containers and transported in ice to the laboratory before testing. Water was collected 15 cm below the surface and at least half a meter away from the shore of a dam or river.

All samples were kept in a refrigerated condition until tested and all samples were tested within 10 days of collection with the exception of fresh produce samples that were tested within 24 hours of collection.

Procedure for testing: Some samples were tested at the University of Melbourne and some samples were tested by Silliker-Microtech. Tests specified were *L. monocytogenes* detection and enumeration.

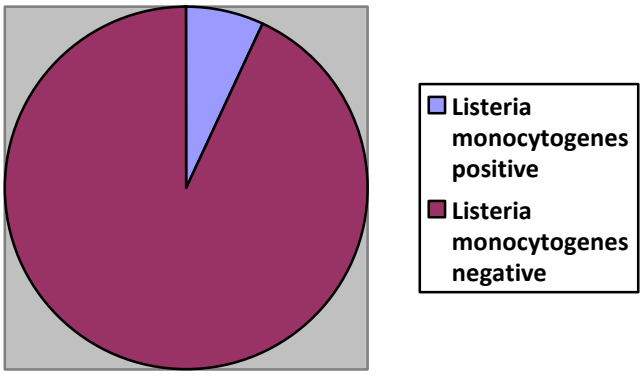
7. First year testing results (2008-2009)

7.1 Summer testing:

Summer testing was carried out on a number of vegetable farms in Victoria and Queensland; testing for *L. monocytogenes* was carried out by conventional standard method (full enumeration). Testing was carried out in the weeks of January 5th to the 9th, February 9th to the 13th and March 16th to the 20th in Victoria and in the weeks of February 16th to 20th and March 23rd to the 27th in Queensland. A total of 331 tests were carried out on leafy vegetables, 98 tests on soil, 148 tests on animal manure and 73 tests on water.

Water Testing:

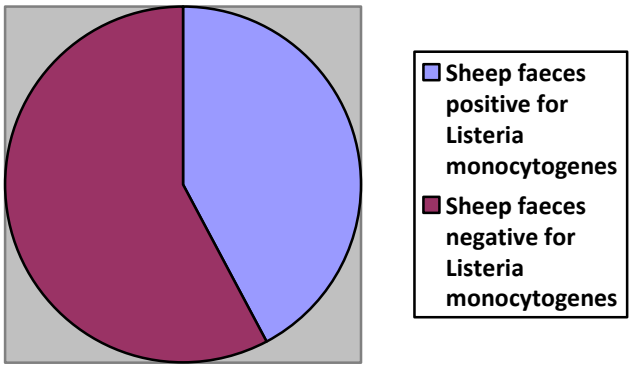
L. monocytogenes was found in very low levels in 5 of the 73 water samples (at less than 100 CFU/100 ml).



Overall, of the 73 water samples tested, only 5 were positive for *L. monocytogenes* at less than 10 CFU per 100 ml. This corresponds to a figure of 6.8% of all water samples containing detectable levels of *L. monocytogenes.*

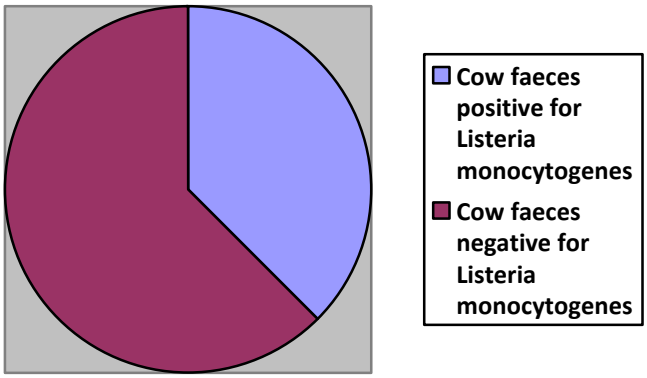
Manure testing:

Faeces samples originating from sheep (total of 45 samples) showed that 19 were positive for *L. monocytogenes*.



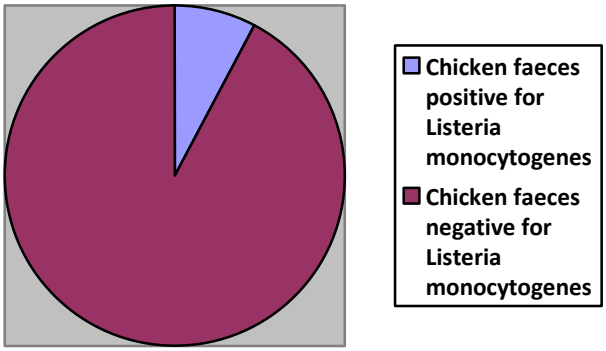
Overall out of the 45 samples of ovine faeces tested, 19 were positive for *L. monocytogenes* at levels of 100 CFU/g or more. This corresponds to a figure of 42.2% of all ovine faecal samples containing high levels of *L. monocytogenes*.

Manure samples from bovine sources (total of 32) showed that 12 were positive for *L. monocytogenes*.



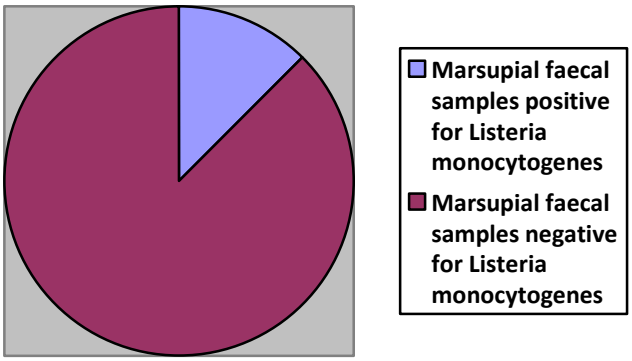
Overall, out of the 32 samples of bovine faeces tested, 12 were positive for *L.monocytogenes* at levels of 100 CFU/g or more. This corresponds to a figure of 37.5% of all bovine faecal samples containing high levels of *L. monocytogenes.*

Manure samples from chickens (39) showed that only 3 were positive for *L. monocytogenes*.



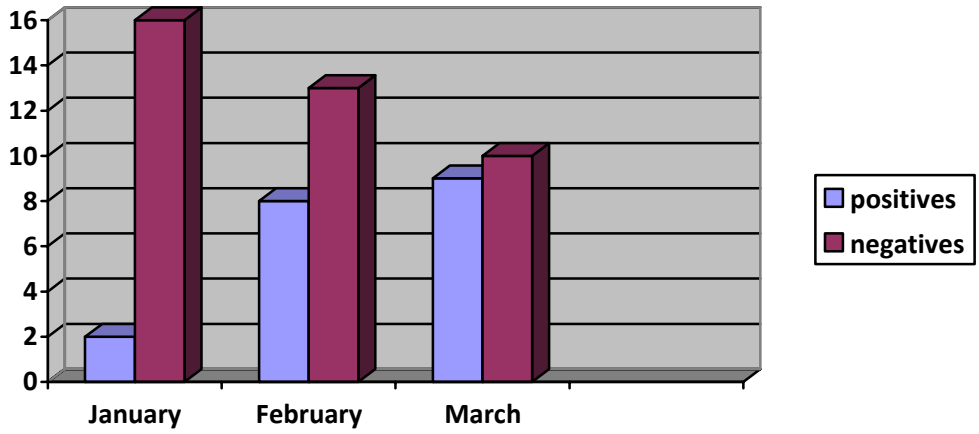
Overall, out of the 39 samples of chicken faeces tested, 3 were positive for *L. monocytogenes* at levels of less than 100 CFU/g. This corresponds to a figure of 7.7% of all chicken faecal samples containing low levels of *Li. monocytogenes.*

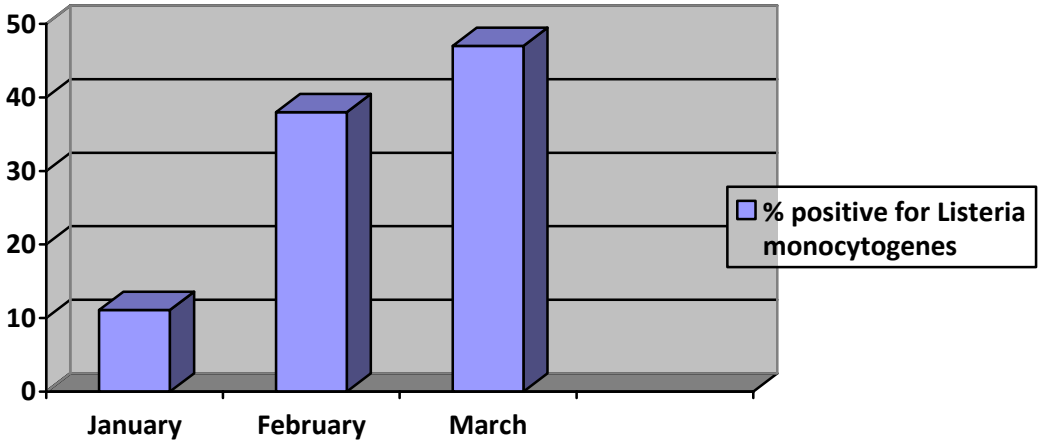
Kangaroo and possum faecal samples (32) showed that 4 were positive for *L. monocytogenes*, corresponding to a figure of 12.5% of all marsupial faecal samples containing low levels of *L. monocytogenes.*



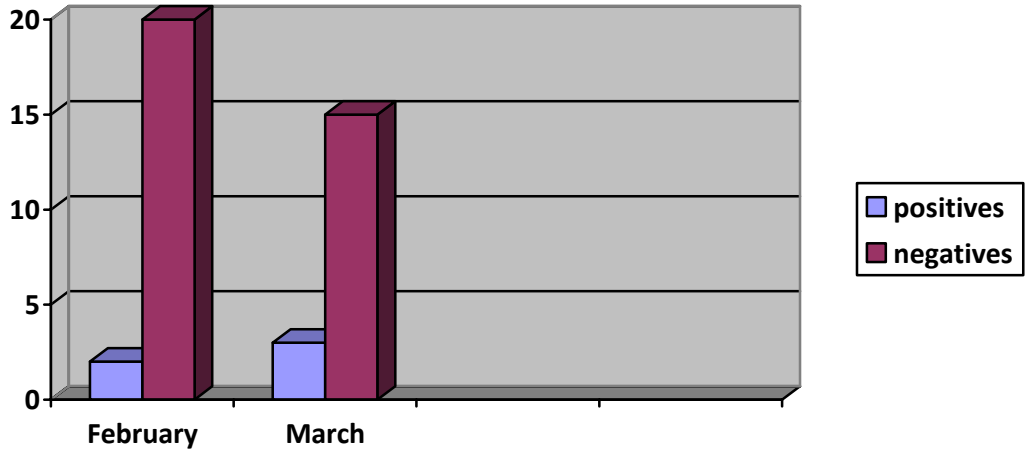
Soil testing:

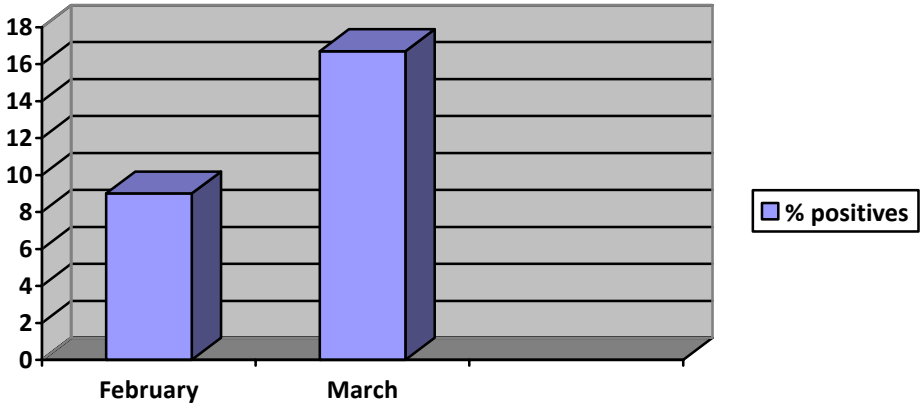
Soil samples tested in Victoria showed that in March the incidence of *L. monocytogenes* was higher (9 out of 19) than in February (8 out of 21) or January (2 out of 18).





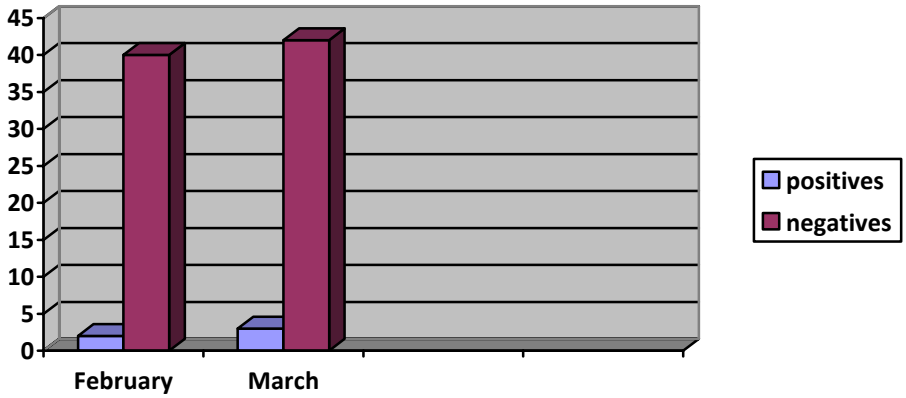
In Queensland, soil tests remained relatively stable with 2 out of 22 soil samples positive for *L. monocytogenes* in February and 3 out of 18 soil samples being positive for *L. monocytogenes* in March.

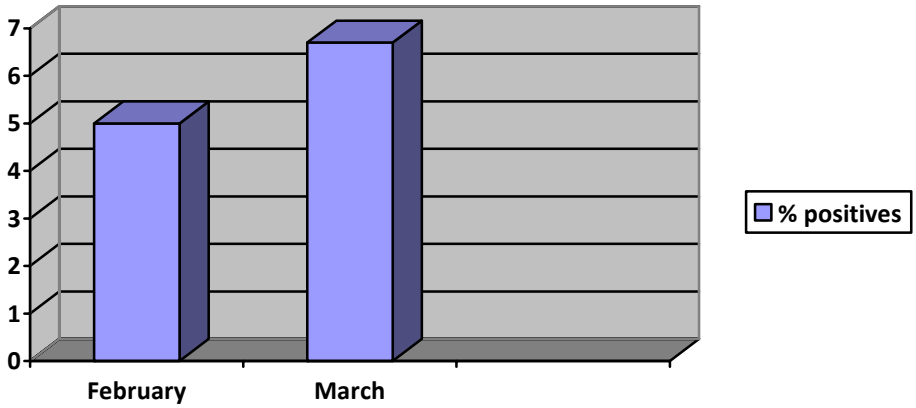




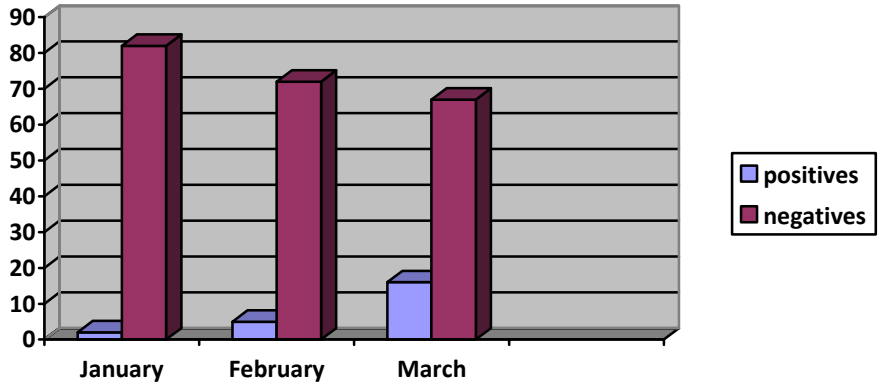
Produce testing:

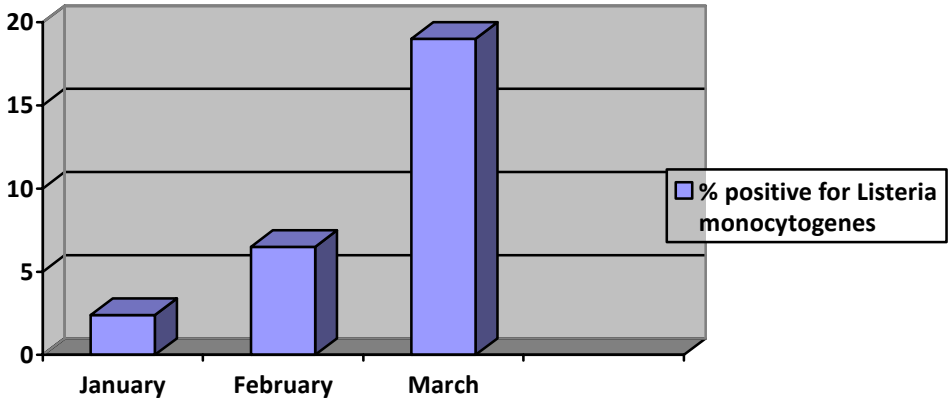
Produce samples from Queensland showed that 2 were *L. monocytogenes* positive at harvest out of 42 samples tested in February, 3 out of 45 samples tested were positive for *L. monocytogenes* in March. There had been a lot of rain over the two months and there were no days with wind speed over 60 km/h (87 samples were tested in total).





In Victoria, 2 samples out of 84 tested were positive for *L. monocytogenes* in January, 5 samples out of 77 were positive for *L. monocytogenes* in February and 16 samples out of 83 were positive for *L. monocytogenes* in March (244 samples tested in total).





A Chi-squared test was conducted on the Victorian results, as shown in the following table, and it was highly significant (*P*=0.001).

	Year 1		
Victoria	January	February	March
positive	2	5	16
negative	82	72	67
% positive	2%	6%	19%

A Chi- squared test on the results for January, February and March in Victoria showed highly significant results (P=0.0001) for the increased levels of *L. monocytogenes* in produce from January to March in Victoria.

7.2 Discussion of results:

The first observation from these results is that from the total number of water samples tested, a small portion showed relatively low levels of *L. monocytogenes* contamination. Only 5 of the 73 water samples tested were positive (see table 1 in Appendix 1). This corresponded to 6.8% of all water samples tested being positive for *L. monocytogenes*.

Bovine and ovine samples with levels of detectable *L. monocytogenes* were 37.5% and 42.2% respectively. The levels of *L. monocytogenes* found in ruminants is similar to that found in Northern Spain (Estaban J. *et al.* 2009), a country with a climate similar to Eastern Australia. Marsupial faecal samples with detectable levels of *L. monocytogenes* were at 12.5%. These levels were less that those detected in ruminants but no references were found as to the status of marsupials as shedders of *L. monocytogenes.*

Soil testing showed a progressive increase in detection for each of January, February and March. By the end of March, *L. monocytogenes* could be detected in over 45% of soil samples tested in Victoria. The figure for Queensland for the months of February and March were much lower, with only 9 and 17% of soils having detectable levels of *L. monocytogenes,* respectively.

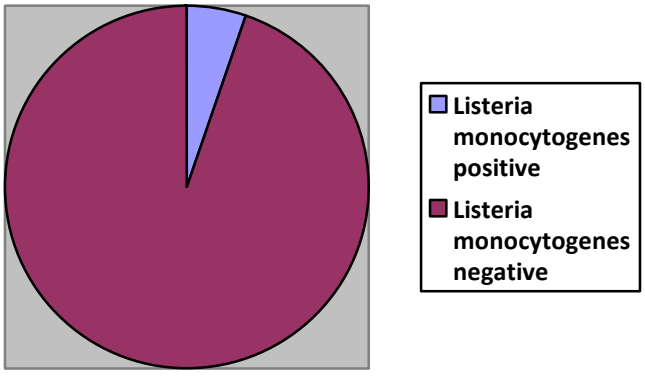
7.3 Winter testing:

Three sampling dates were used to collect samples from the same farms that were sampled in summer. Soil/manure/water/produce was tested in the same manner as it was tested in summer and results were evaluated at the end of the sampling process. Dates in June, July and August were used to collect the samples. One of the farms in Queensland was not operating at the time of sampling and produce was taken from farms close by.

Results:

Water Testing:

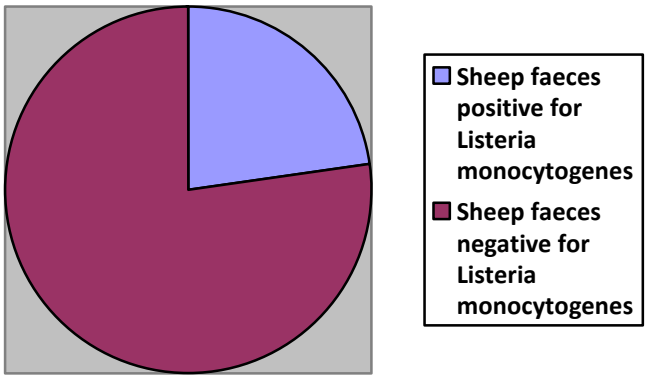
L. monocytogenes was found in very low levels in 3 of the 56 water samples (at less than 100 CFU/100 ml).



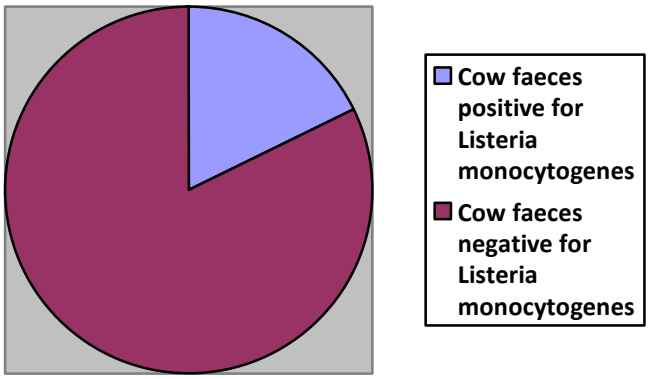
Overall, of the 56 water samples tested, only 3 were positive for *L. monocytogenes* at less than 10 CFU per 100 ml. Some of the water supplies tested in summer were not operating in winter, hence a total lower number of samples were tested. The figures correspond to 5.4% of all water samples containing detectable levels of *L. monocytogenes.*

Manure testing:

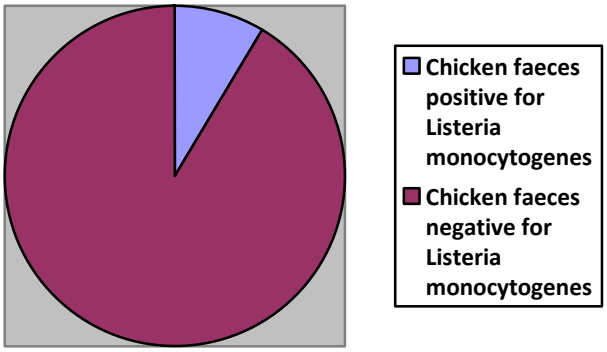
Faecal samples from sheep tested between June and August showed that out of 35 tested 8 were positive for *L. monocytogenes.*



Cow faecal samples tested showed that out of 28 samples tested, 5 were positive for *L. monocytogenes*.



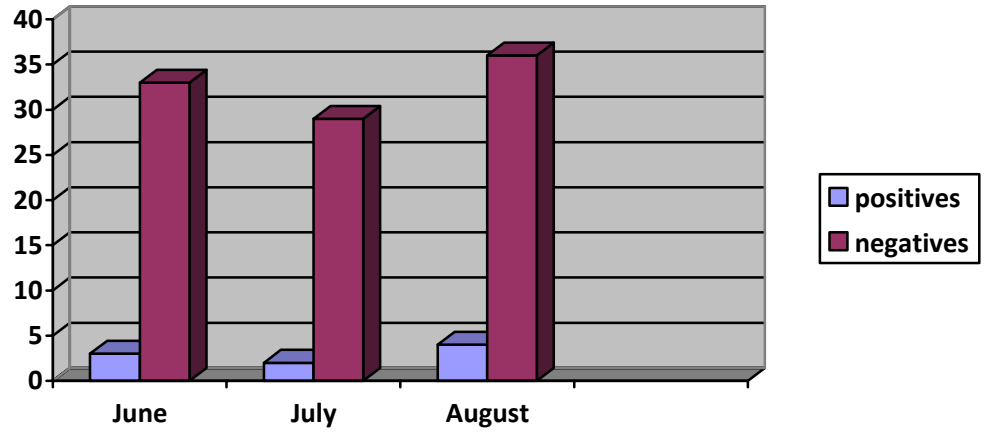
Manure samples from chickens showed that only 4 were positive for *L. monocytogenes* from the 46 samples taken, some of these samples were aged manure samples.

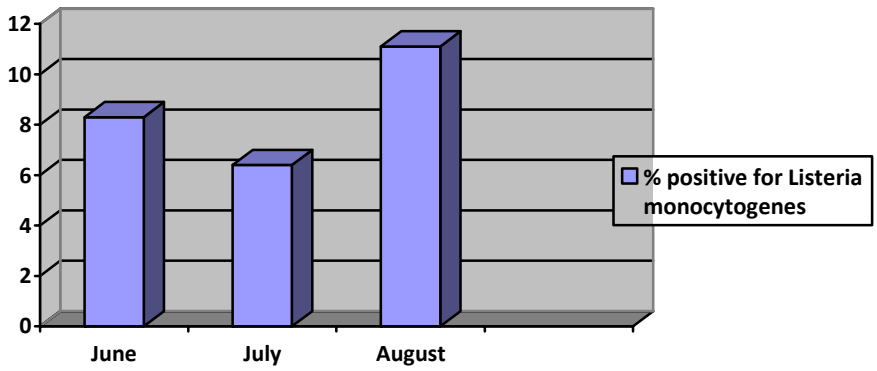


Overall, out of the 46 samples of chicken faeces taken during the months of June to August 2009, 4 were positive for *L. monocytogenes* at levels of 100 CFU/g or less. This corresponds to a figure of 8.7% of all chicken faecal samples containing low levels of *L. monocytogenes*.

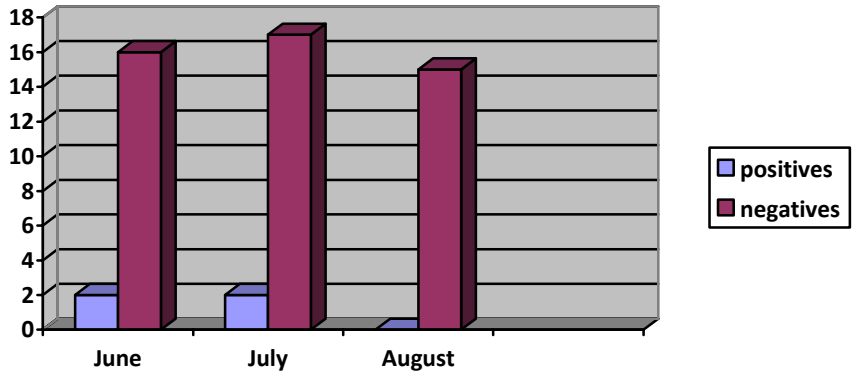
Soil sampling:

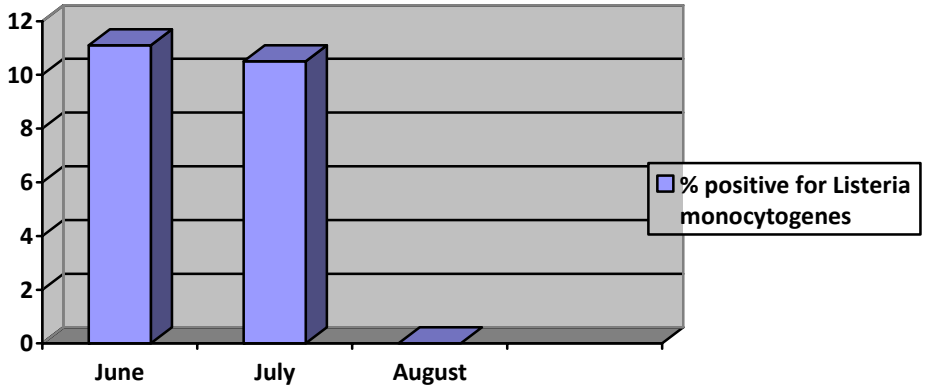
Soil samples from Victoria between June and August showed that out of 103 samples of soil tested 9 had detectable levels of *L. monocytogenes* at less than 100CFU/g of soil.





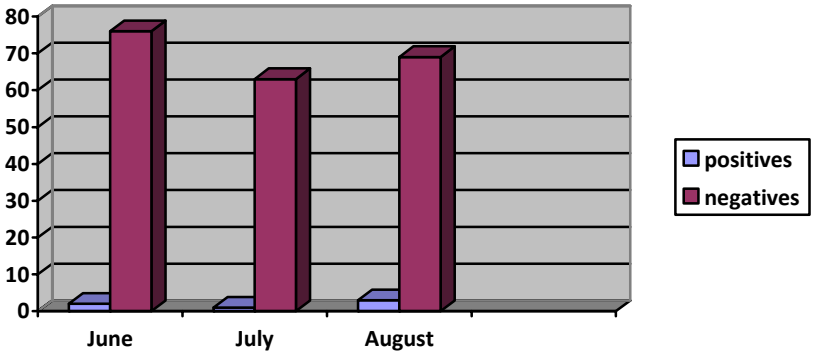
Soil samples from Queensland between June and August showed that out of 52 samples of soil tested, 4 had detectable levels of *L. monocytogenes* at less than 100 CFU/g of soil.

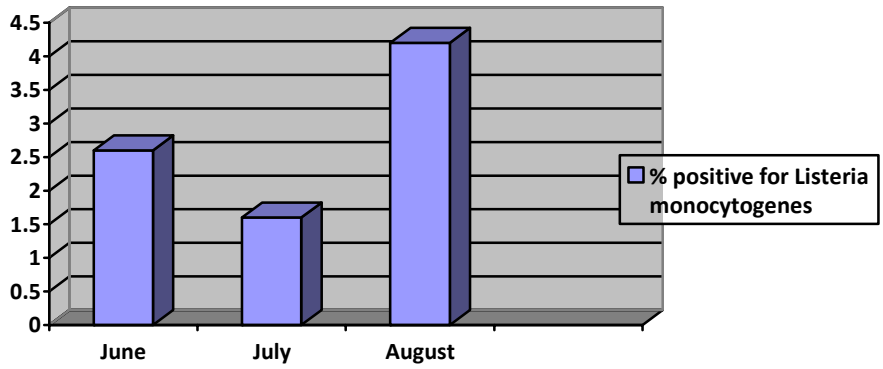




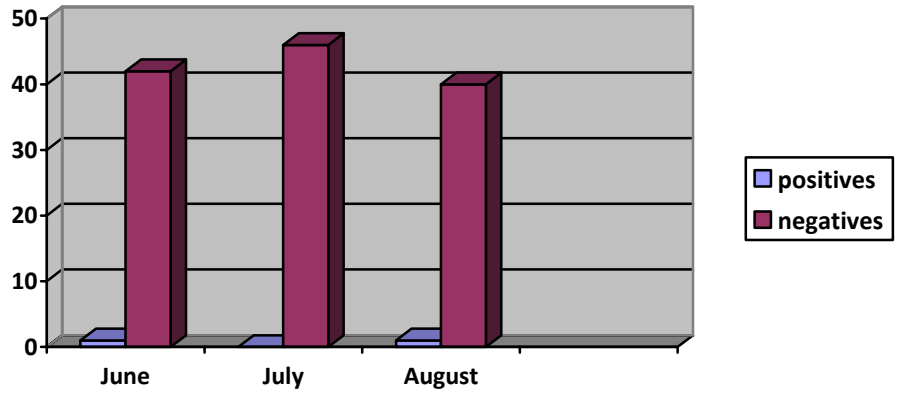
Produce testing:

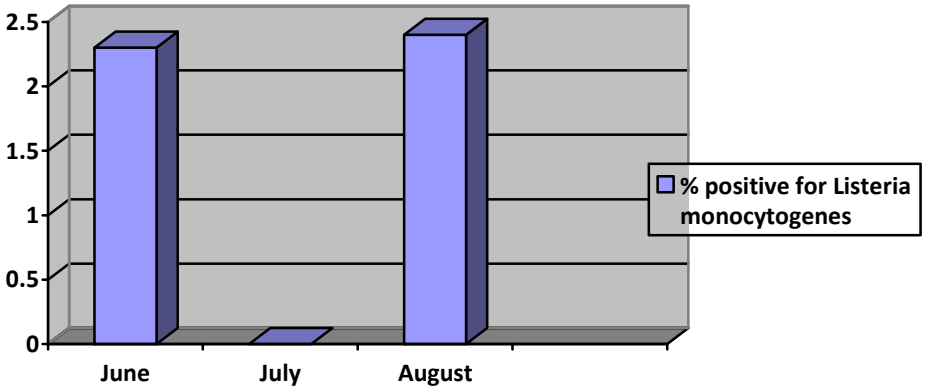
A total of 214 samples of produce were tested between June and August in Victoria, only 6 were positive for *L. monocytogenes*. Two out of 78 in June, one out of 64 in July and three out of 72 in August.





A total of 130 samples of produce were tested between June and August in Queensland and only two were positive for *L. monocytogenes*, one out of 43 samples tested in June, none out of 46 samples tested in July and one out of 41 samples tested in August.





7.4 Discussion of results:

The results from the two season testing after the first year suggests that the presence of *L. monocytogenes* on produce is much lower during the winter months than in the summer months. The results also support the observation that *L. monocytogenes* is more of an issue in Victoria than in Queensland. These two findings are important for the vegetable industry as

they show that on farm *Listeria* management is far more important in the summer months than in the winter months and that Victorian growers need to be more pro-active in *Listeria* management than growers in Queensland.

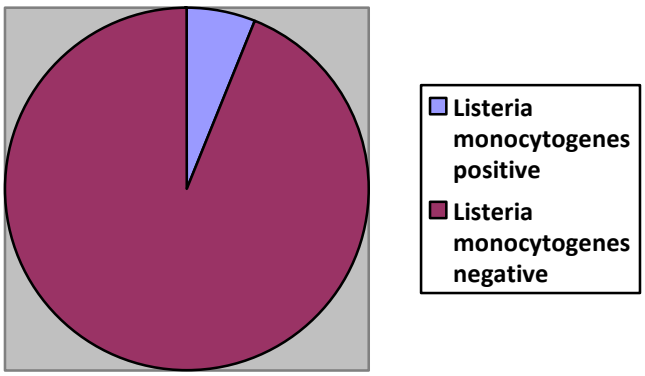
8. Second year testing results (2009-2010)

8.1 Summer testing:

Summer testing was carried out on a number of vegetable farms in Victoria and Queensland as per the previous year. Testing for *Listeria monocytogenes* was carried out by conventional standard method (full enumeration). Testing was carried out in the weeks of January 4th to the 7th, February 8th to the 11th and March 9th to the 12th in Victoria and in the weeks of February 22nd to 24th and March 16th to the 19th in Queensland. Water tests were carried out as in the previous year, not all water sources that were tested the previous summer were available and hence the differences in numbers, overall 65 water samples were tested. Manure samples were selected during the same times and tested; overall 133 manure samples were taken. A total of 293 samples were carried out on leafy vegetables and 145 on soil.

Water testing:

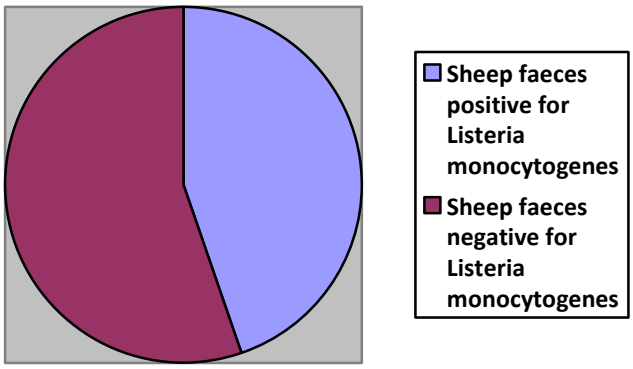
L. monocytogenes was found in very low levels in 4 of the 65 water samples (at less than 100 CFU/100 ml).



Overall of the 65 water samples tested only 4 were positive for *L. monocytogenes* at less than 10 CFU per 100 ml. This corresponds to a figure of 6.2% of all water samples containing detectable levels of *L. monocytogenes*.

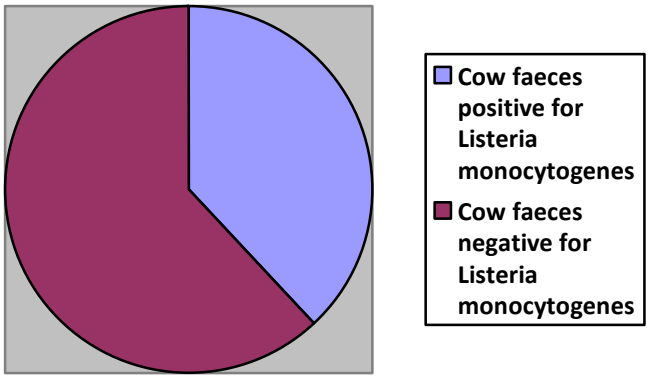
Manure testing:

Faecal samples originating from sheep (total of 38 samples) showed that 17 were positive for *L. monocytogenes*.



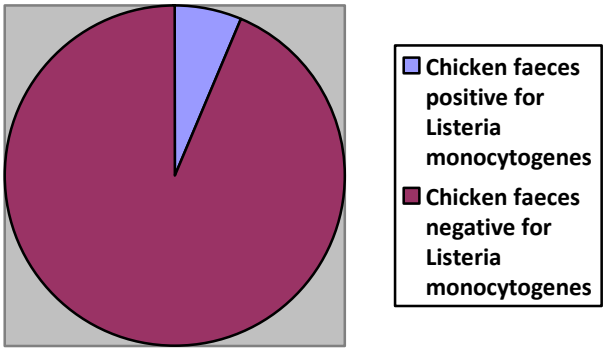
Overall, out of the 38 samples of ovine faeces 17 were positive for *L. monocytogenes* at levels of 100 CFU/g or more. This corresponds to a figure of 44.7% of all ovine faecal samples containing high levels of *L. monocytogenes.*

42 manure samples from bovine sources showed that 16 were positive for *L. monocytogenes*.



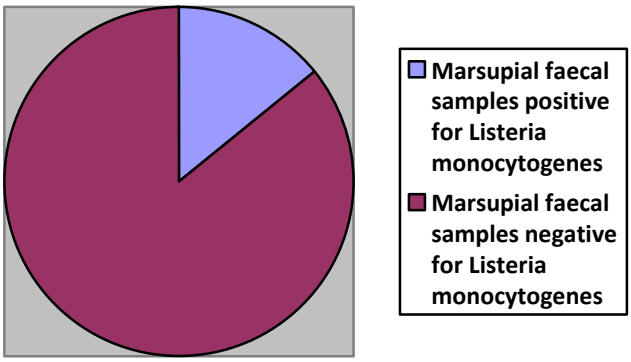
Overall out of the 42 samples of bovine faeces tested, 16 were positive for *L. monocytogenes* at levels of 100 CFU/g or more. This corresponds to a figure of 38.1% of all bovine faecal samples containing high levels of *L. monocytogenes.*

29 manure samples from chickens were tested and the results showed that only 2 were positive for *L. monocytogenes*.



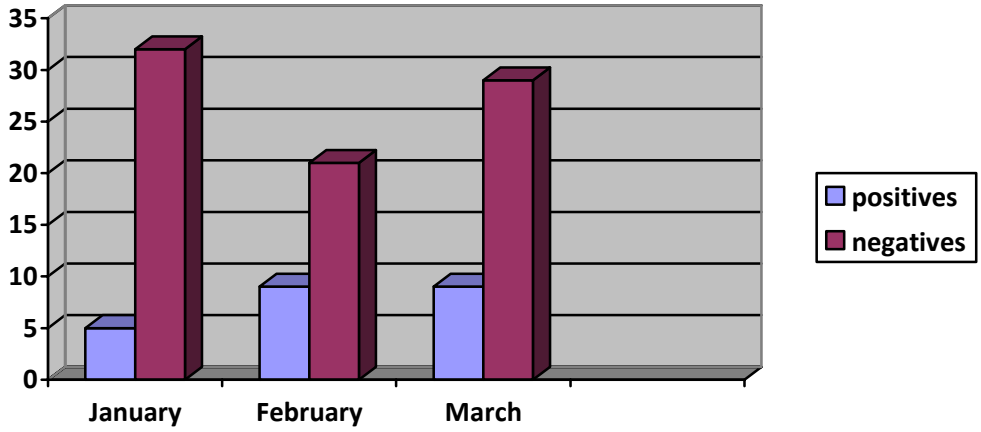
Overall, out of the 29 samples of chicken faeces tested, 2 were positive for *L. monocytogenes* at levels of 100 CFU/g or less. This corresponds to a figure of 6.9% of all chicken faecal samples containing low levels of *L. monocytogenes.*

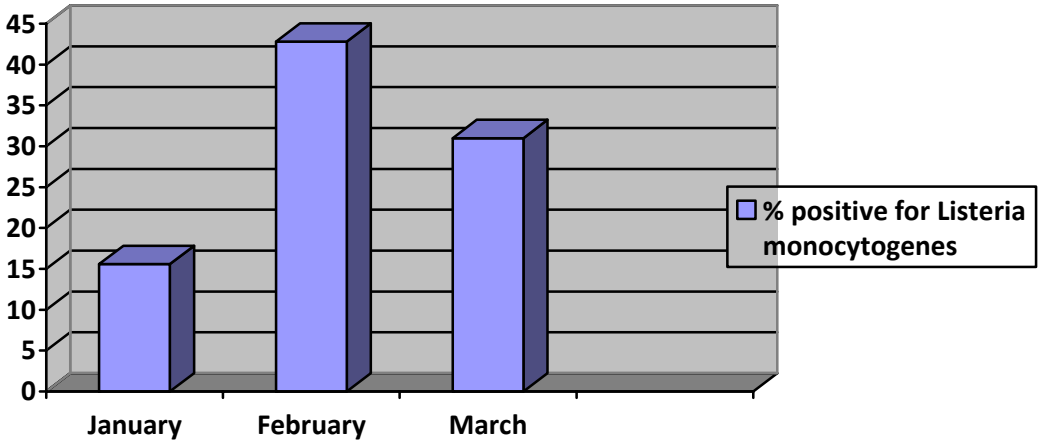
24 Kangaroo and possum faecal samples were tested and the results show that 4 were positive for *L. monocytogenes*.



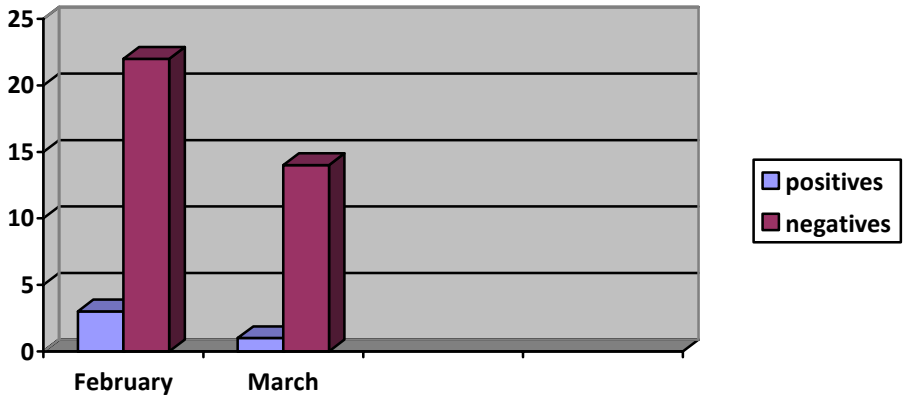
Soil sampling:

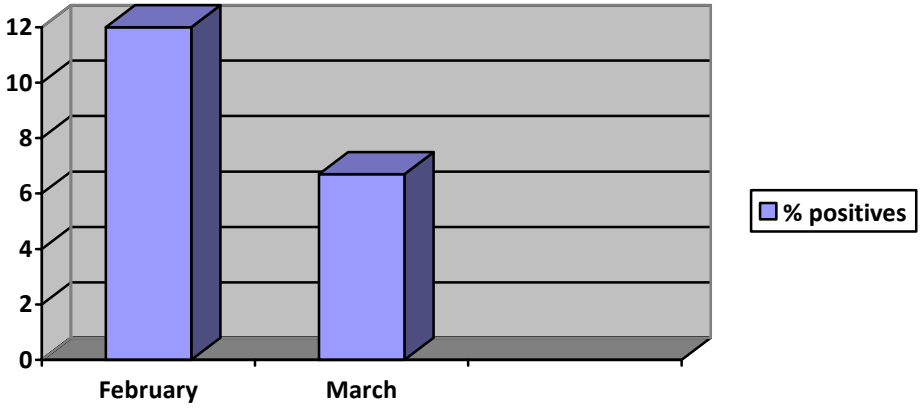
Soil samples tested showed that in Victoria, the prevalence of *L. monocytogenes* was higher in February (9 out of 30) than in March (9 out of 38) or January (5 out of 37).





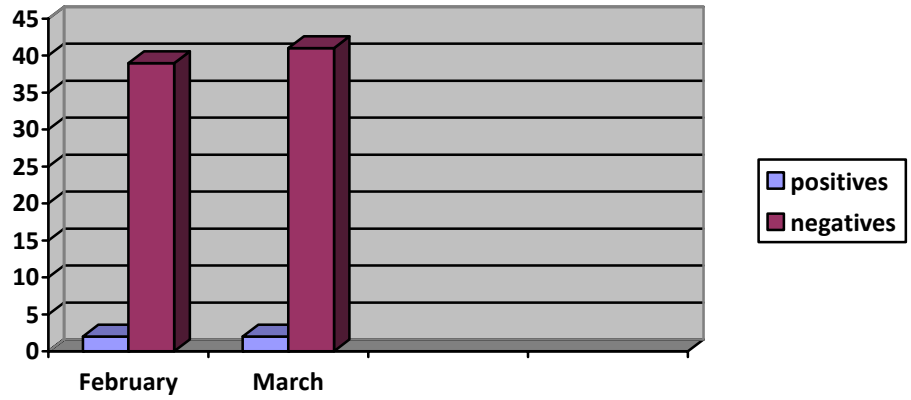
In Queensland soil tests remained relatively stable with 3 out of 25 soil samples positive for *L. monocytogenes* in February and 1 out of 15 soil samples being positive for *L. monocytogenes* in March.

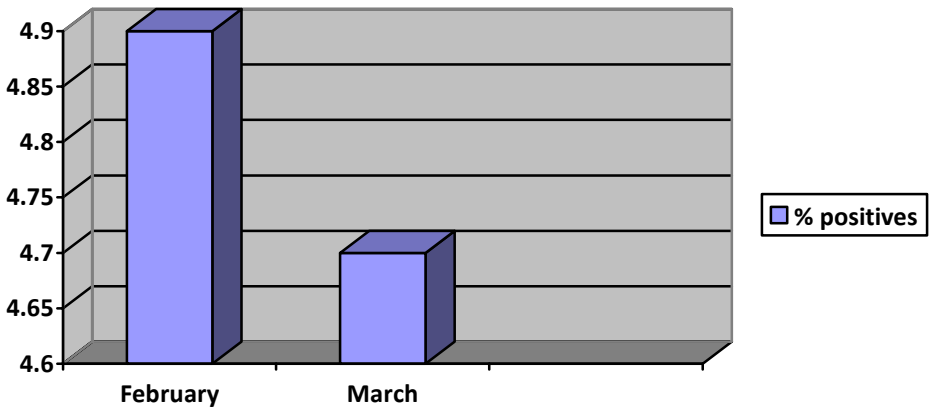




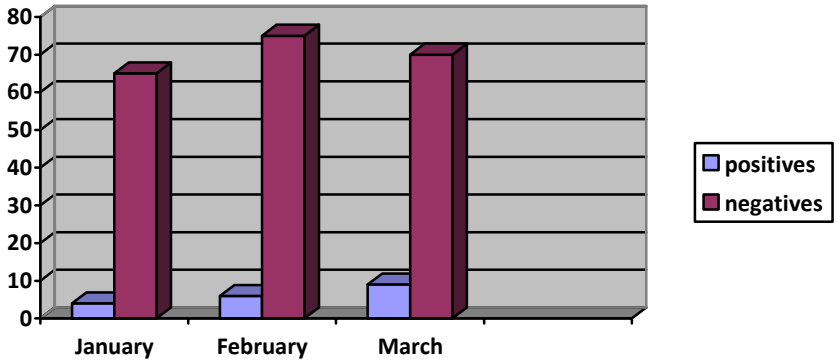
Produce testing:

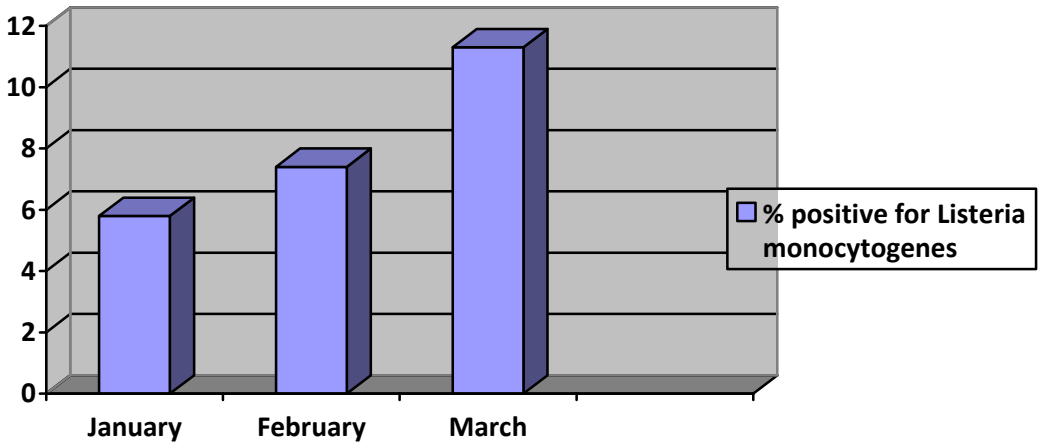
Produce samples from Queensland showed that 2 were *L. monocytogenes* positive at harvest out of 41 samples tested in February, 2 out of 43 samples tested were positive for *L. monocytogenes* in March.





In Victoria, 4 samples out of 69 tested were positive for *L. monocytogenes* in January, 6 samples out of 81 were positive for *L. monocytogenes* in February and 9 samples out of 79 were positive for *L. monocytogenes* in March.





8.2 Discussion of results:

The first observation from these results is that water samples tested showed relatively low levels of *L. monocytogenes* contamination. Only 4 of the 65 water samples tested were positive (see table 1 in Appendix 1). This corresponded to 6.2% of all water samples tested being positive for *L. monocytogenes*. The levels of *L. monocytogenes* detected in these samples were relatively low.

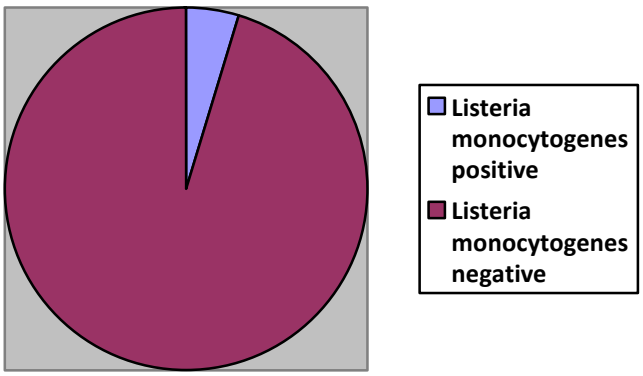
Bovine and ovine samples with levels of detectable *L. monocytogenes* were 38.1% and 44.7 % respectively. The levels of *L. monocytogenes* found in ruminants is similar to that found in Northern Spain (Estaban J. *et al.* 2009), a country with a climate similar to Eastern Australia. Marsupial faecal samples with detectable levels of *L. monocytogenes* were at 16.7 %. These levels were less that those detected in ruminants but no references were found as to the status of marsupials as shedders of *L. monocytogenes.*

Soil testing showed an increase in the percentage of samples testing positive for *L. monocytogenes* in the month of February for both Victorian and Queensland soil samples. *L. monocytogenes* could be detected in over 21.9% of soil samples tested in Victoria. The figure for Queensland for the months of February and March were much lower, with only 10% of soils having detectable levels of *L. monocytogenes*.

8.3 Winter testing:

Water Testing:

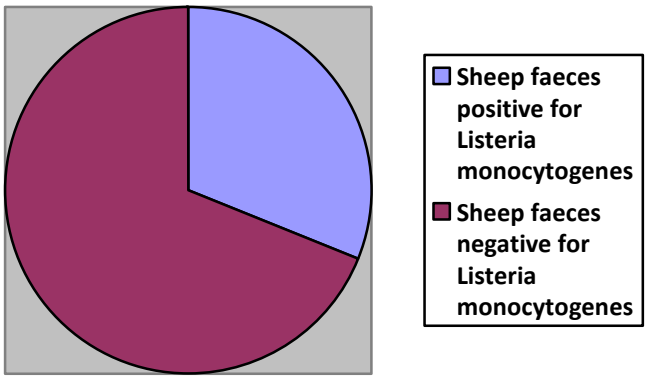
L. monocytogenes was found in very low levels (less than 100CFU/g) in 3 of the 62 water samples.



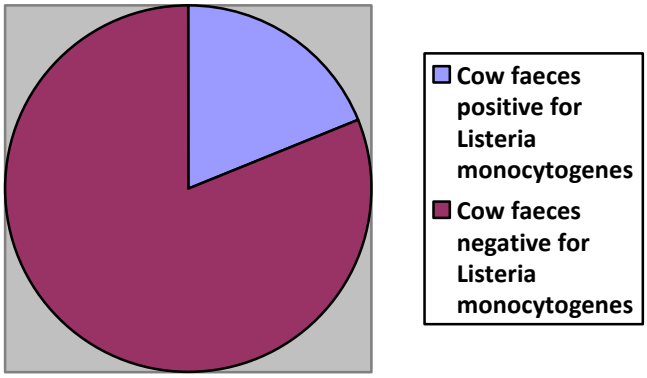
Overall, of the 62 water samples tested, only 3 were positive for *L. monocytogenes* (at less than 10 CFU per 100 ml). Some of the water supplies tested in summer were not operating in winter, hence the total number of samples tested was lower in winter. The figures correspond to 4.8% of all water samples containing detectable levels of *L. monocytogenes.*

Manure testing:

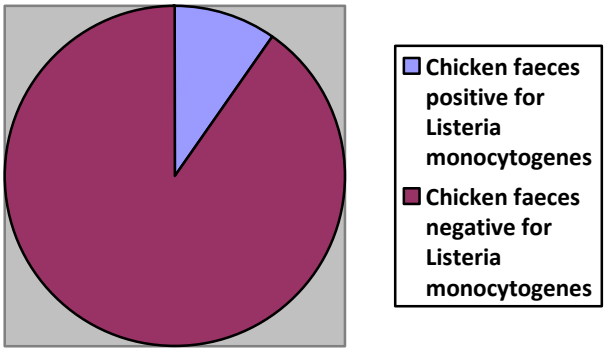
Faecal samples from sheep tested between June and July showed that out of 29 tested, 9 were positive for *L. monocytogenes* which equates to 31.0% of samples testing positive.



Cow faecal samples showed that out of 32 samples tested 6 were positive for *L. monocytogenes* which equates to 18.8% of samples being positive.



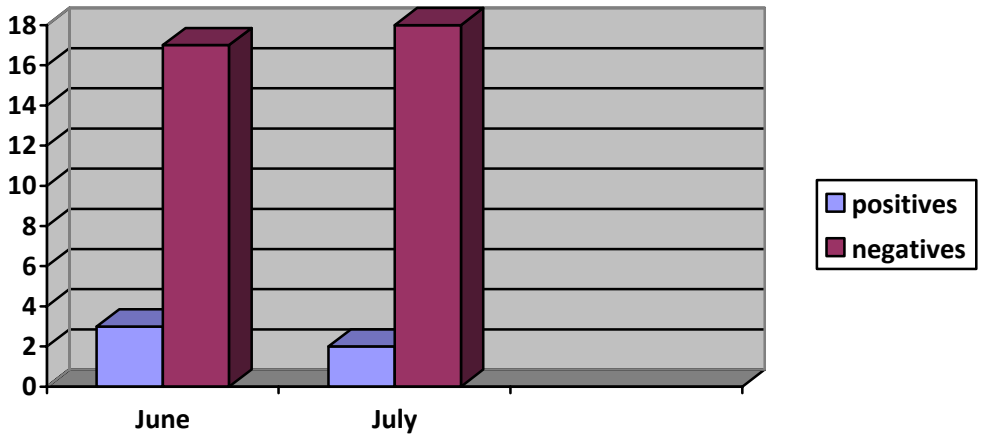
Manure samples from chickens showed that only 3 were positive for *L. monocytogenes* from the 31 samples taken, some of these samples were aged manure samples.

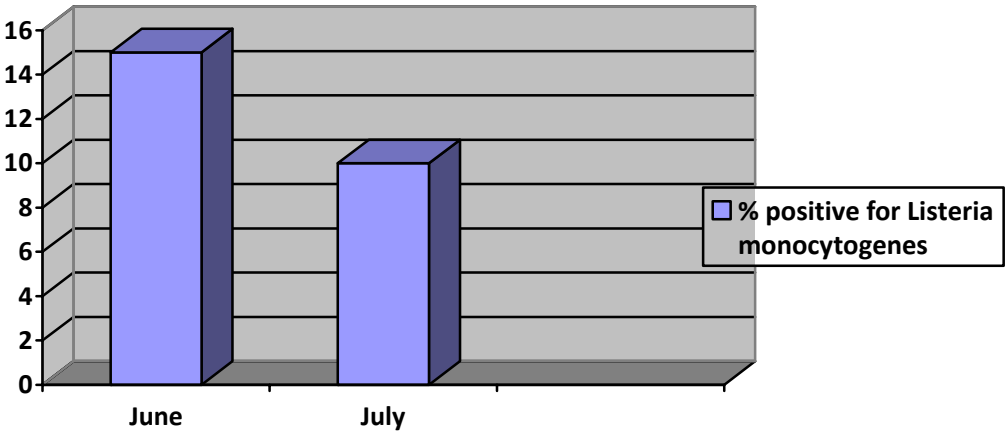


Overall, out of the 31 samples of chicken faeces taken during the months of June to July 2010, 3 were positive for *L. monocytogenes* at levels of 100 CFU/g or less. This corresponds to a figure of 9.7% of all chicken faecal samples containing low levels of *L. monocytogenes*.

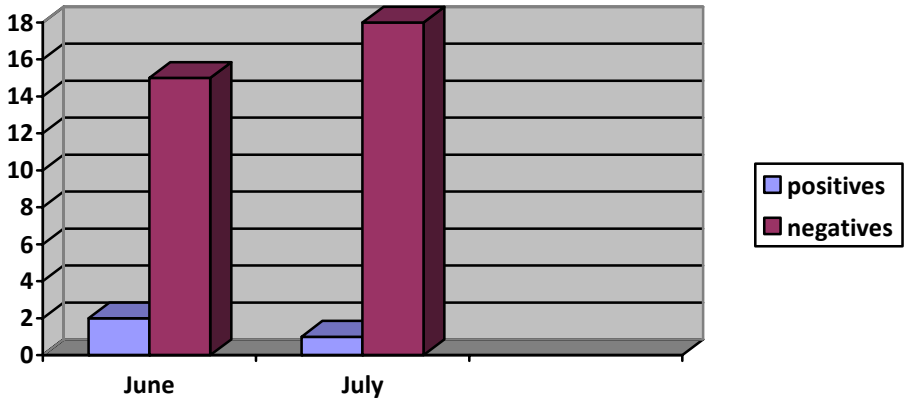
Soil sampling:

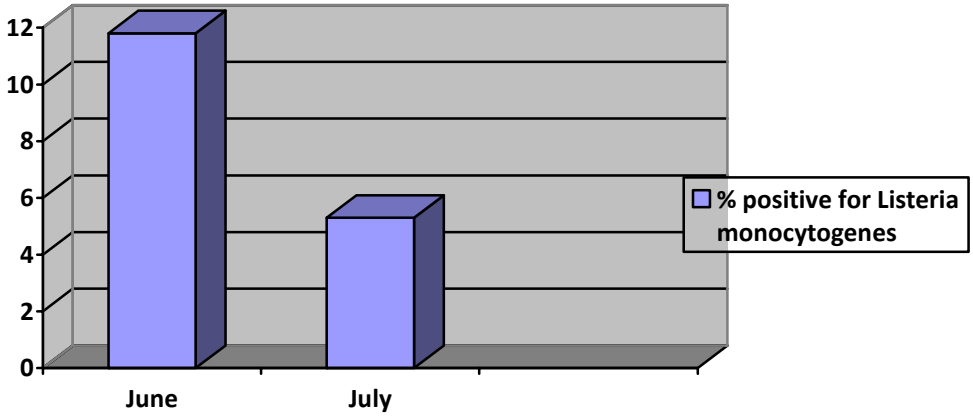
Soil samples taken from Victoria between June and July showed that out of 40 samples of soil tested, 5 had detectable levels of *L. monocytogenes* (at less than 100 CFU/g of soil), equating to 12.5%.





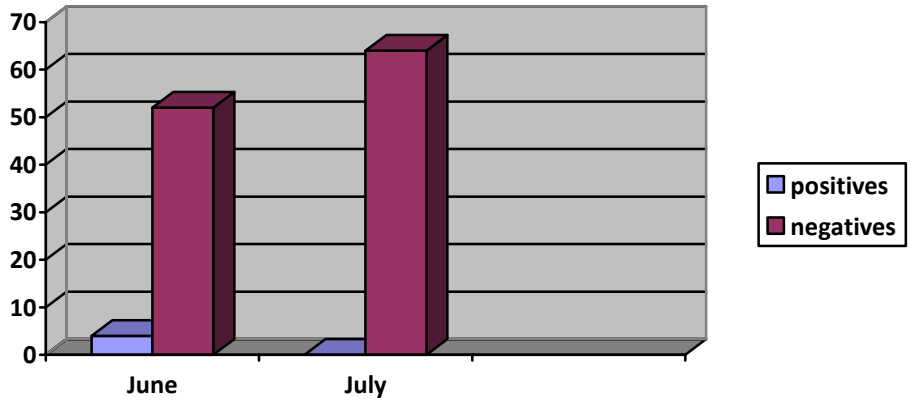
Soil samples from Queensland taken between June and July showed that out of 36 samples of soil tested, 3 had detectable levels of *L. monocytogenes* (at less than 100cfu/g of soil), equating to an overall figure of 13.8%.

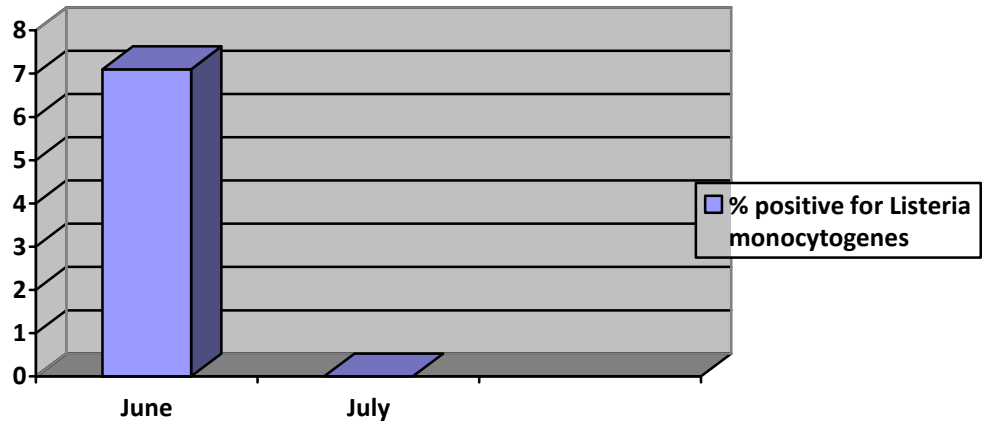




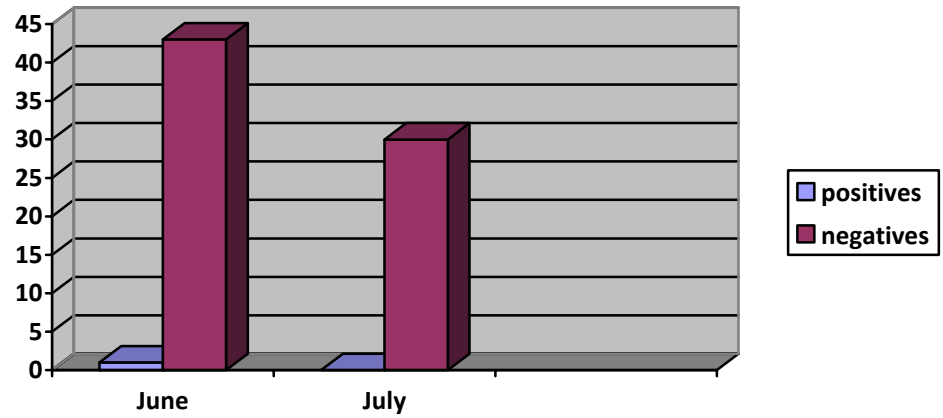
Produce testing:

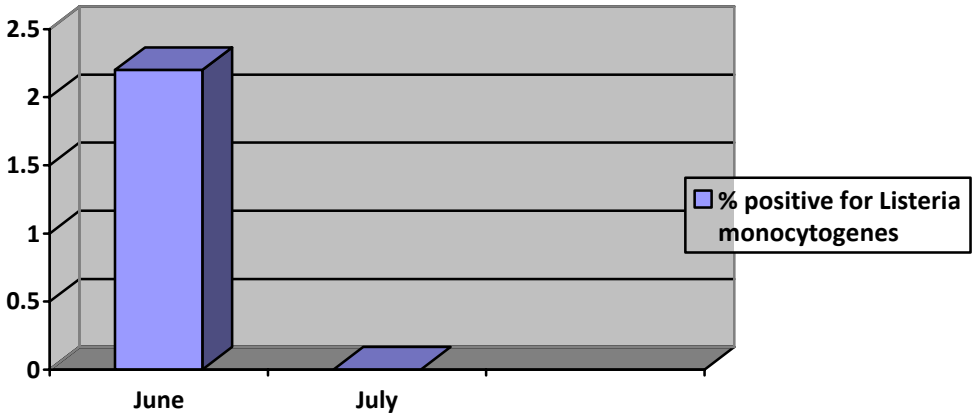
A total of 120 samples of produce were tested between June and July in Victoria, only 4 were positive for *L. monocytogenes*, four in June and none in July, a total of 3.3% testing positive.





A total of 74 samples of produce were tested between June and July in Queensland and only one was positive for *L. monocytogenes,* one in June and none in July, equating to a total of 1.4% testing positive.





8.4 Discussion of results:

L. monocytogenes was found in very low levels in 3 of the 62 water samples (at less than 100 CFU/100 ml).

Faeces samples originating from sheep (total of 29 samples) showed that 9 were positive for *L. monocytogenes*. Manure samples from bovine sources (total of 32) showed that 6 were positive for *L. monocytogenes*. Manure samples from chickens (31) showed that only 3 were positive for *L. monocytogenes*.

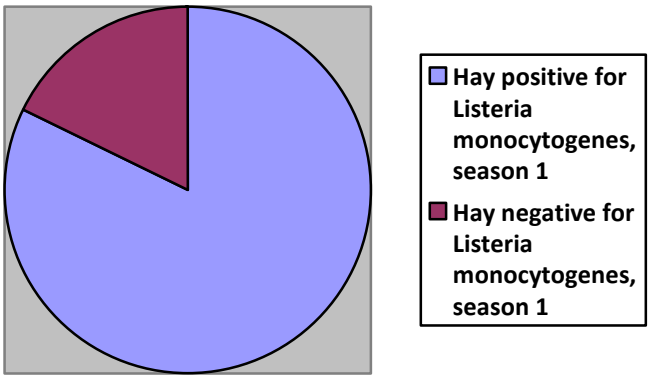
Soil samples tested showed that in Victoria *L. monocytogenes* counts were higher (3 out of 20) in June than in July (2 out of 20). In Queensland soil tests remained relatively stable with 2 out of 17 soil samples positive for *L. monocytogenes* in June and 1 out of 19 soil samples being positive for *L. monocytogenes* in July.

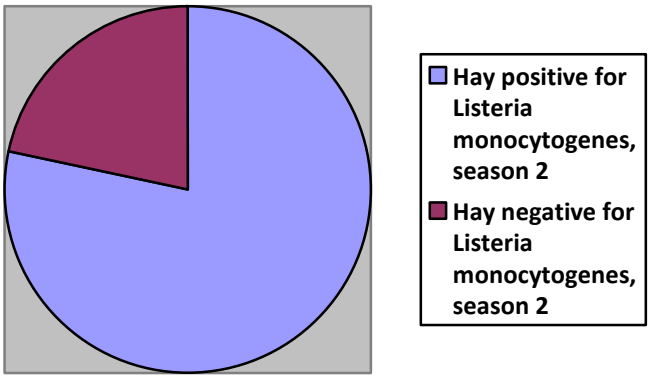
Produce samples from Queensland showed that one was *L. monocytogenes* positive at harvest out of 44 samples tested in June, 0 out of 30 samples tested were positive for *L. monocytogenes* in July.

In Victoria, 4 samples out of 56 tested were positive for *L. monocytogenes* in June, 0 samples out of 64 were positive for *L. monocytogenes* in July.

8.5 Testing of bales of hay (season one and season two):

Bales of hay from the previous season were tested in both the summer of the first season and in the summer of the second season. Bales tested in the first season showed that out of 17 sampled, 14 had detectable *L. monocytogenes*. Bales tested in the second season showed that out of 23 tested 18 had detectable *L. monocytogenes* at a level greater than 100 CFU/g. Samples were collected from deep inside the bale, usually the sample was moist and contained decomposing hay.





9. General Discussion:

9.1 Rapid testing for *L. monocytogenes*:

The polymerase chain reaction (PCR) was the first and remains the most widely applied rapid DNA based testing process in diagnostic laboratories. PCR is a fast and rapid way to quickly amplify specific sequences of target DNA from micro-organisms, including *L. monocytogenes*, to an amount that can be visualized with a variety of detection devices. PCR employs two oligonucleotide primers that flank the beginning and end of a specific DNA sequence, a thermostable enzyme (DNA polymerase) that is capable of synthesizing the specific DNA, and double-stranded DNA to function as a template for DNA polymerase.

The PCR process begins at a high temperature (e.g. 95°C) to denature and open the double-stranded DNA, template into single-stranded DNA followed by a relatively low temperature (e.g. 56°C) to enable annealing between the single-stranded primer and the single-stranded template, and then a temperature of 72°C to allow DNA polymerase copying (extension) of the template. The whole process is repeated 25–30 times so that a single copy of DNA template can turn into billions of copies within 3–4 h.

Gel electrophoresis is typically used to detect the amplified product. As the primers bind to specific sites surrounding the target DNA, the size of the amplified product can be anticipated and detected with a DNA stain, as a band of known size on a gel.

This is not the type of rapid test that can be performed outside a specialized laboratory but instead is a test that needs to be performed by trained technicians in a highly specialized laboratory to control contaminants and the potential for false positives. The biggest advantage of PCR is its sensitivity it can be exceptionally sensitive as our results have confirmed, however the specificity could be a problem. It is prone to false positives as it may be too sensitive. In addition, the rapid test is not that rapid when one considers the enrichment steps required to get suitable results. Although the test can be completed in 24 hours, in our hands, due to the longer enrichment step utilized, the test took 48 hours to complete. The same time used by VIDAS, a test that costs a lot less and has similar sensitivity and specificity. PCR testing still has a way to go before it can reach the standard required by Australian Standards. Most importantly the sequence of genes that is used to make the oligonucleotide primers needs to be standardized so that everyone is using the same sequence rather than using different sequences. For this reason alone the recommendation to the Australian horticulture industry is that it continues using NATA approved testing protocols and that any positive be confirmed by the Australian standard method, perhaps the only test fully acceptable in our legal system in case of litigation. Given that most laboratories run rapid screen tests to detect *Listeria* species in a sample, it is important that the industry specifies conformation with Australian Standard methods, or equivalent if these have been approved by NATA.

DNA based testing, including gene segment amplification or PCR technology (polymerase chain reaction) and real-time PCR, is increasingly applied in food diagnostics for the detection of *L. monocytogenes* due to the availability of different specific commercial test methods. However, these methods still require extensive enrichment steps and even selection steps. Microarrays and biosensors are some examples of new technologies that might be used routinely for the detection of *L. monocytogenes* in foods in the future.

Confirmation of the presence of *L. monocytogenes* must be carried out according to Australian Standards procedures. Australian Standard procedure AS 1766.2.16.1-1998: Food microbiology - Examination for specific organisms - Food and animal feeding stuffs - Horizontal method for the detection and enumeration of *Listeria monocytogenes* – This detection method describes a culture method that can take up to seven days to complete.

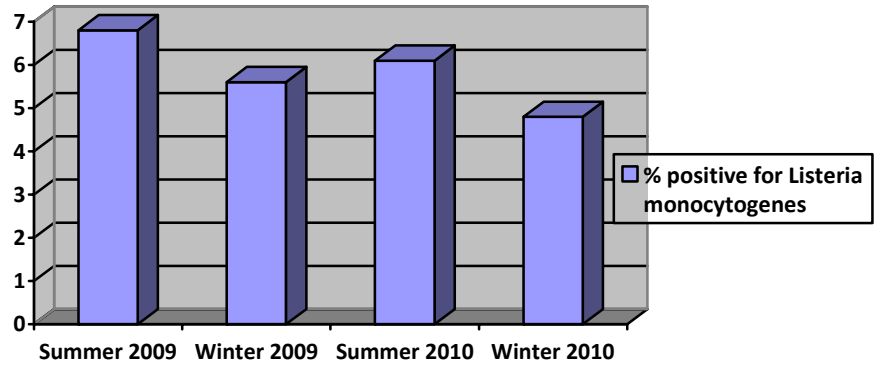
Each of the test procedures has pros and cons, the laboratories must weigh up a number of factors in determining which test they will use. Cost, time and number of manipulations or enrichment steps are often the issues that determine the test of choice. The Australian Standard method for the detection of *L. monocytogenes* lists a culture method that can take between 5 and 7 days, depending on the laboratory. The test requires a number of manipulations and hence is not widely used by routine testing laboratories. A grower needs to request this method for confirmation and enumeration of *L. monocytogenes*. Since working

on this PCR based test, a number of tests have been described in the literature that may provide a better fit for the horticultural industry (Jeyaletchumi Ponniah *et al* 2010).

9.2 *L. monocytogenes* in vegetable farms

Water Testing

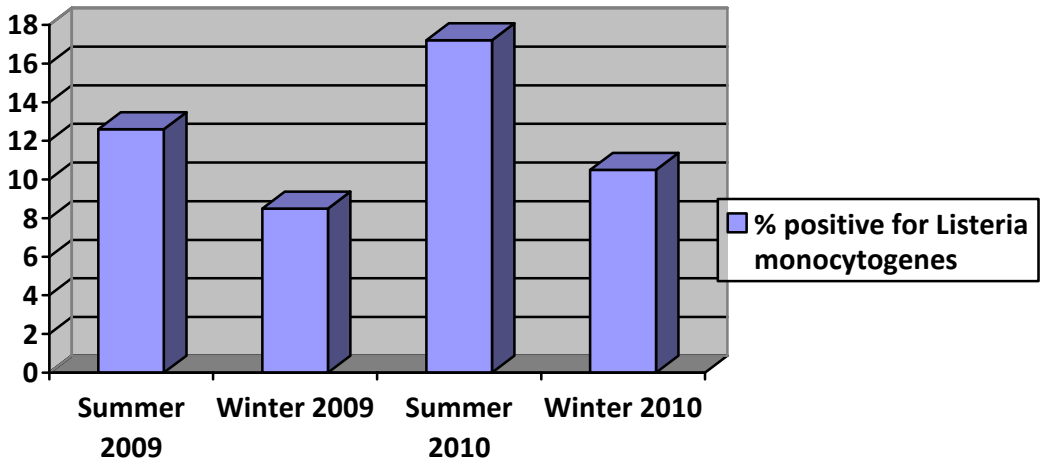
Water testing conducted over two years in summer and in winter in each year suggests that *L. monocytogenes* is not often detected in irrigation water sources either in summer or in winter. Across all four sampling periods there is very little difference between the results. Indications that they are slightly higher in summer than in winter are not supported by statistical analysis. These levels are not too dissimilar from levels reported overseas. Work by E. Lyauter *et.al.* (2007) suggests that *L. monocytogenes* is found at a rate of 10% in catchment waters in Ontario Canada. In the northern Netherlands, *L. monocytogenes* was found in 21% of samples of surface water collected from canals, lakes, ditches, effluent from a sewage treatment plant, canals leading from this sewage treatment plant to the sea, and the sea (Dijkstra, 1982). This latter data however was skewed as it contained sewage treatment plants in the study. Overall the results from this study have not shown that irrigation water used in vegetable farming in Australia is a significant risk in spreading *L. monocytogenes* to leafy vegetables and herbs. Analysis of the data in closer detail shows that most of the detections have been in irrigation channels and rivers (Apendix 2).



In addition, all the detections have been at levels of less than 100 CFU/100 ml. Due to the limits of the test, it was impossible to detect the exact number.

Soil testing

A number of reports indicate that soils are heavily contaminated with *L. Monocytogenes.* Work by Weis and Seelinger (1975) report contamination levels of 8.7% to 51.4% on surface sampling of soils. The results presented in this study have found contamination levels of 8.5% to 10.5% in winter sampling and 12.6% to 17.2% in summer samplings. It is also known that whilst soil is often contaminated with *L.monocytogenes* it does not appear to support the growth of this microbe (Fenlon and Shepherd, 2000). Theories as to how soils become contaminated with *L. monocytogenes* include decaying plant matter and faecal material. One review indicates that faecal shedding of infected domestic and wild animals, including wild birds, may be a more important cause of soil contamination (reviewed by Gray and Killinger, 1966; Fenlon, 1985).

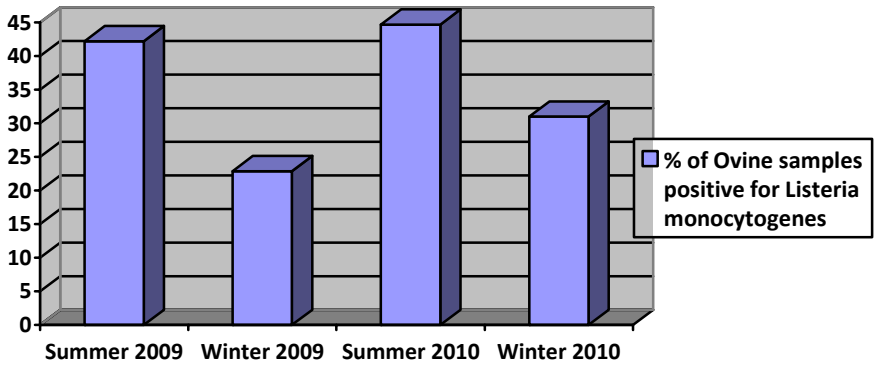


Using a binary logistic regression procedure to model the proportion of positive cases using season and state, we found that the odds of a positive case are 2.6 times higher in summer compared to winter (P<0.001). We are 95% confident that the true odds ratio is between 1.5 and 4.5. The odds of a positive case are 2.0 times higher in Victoria than Queensland (P=0.020). We are 95% confident that the true odds ratio is between 1.1 and 3.7. The results obtained in this study suggest that soil contamination appears to be higher in summer than in winter, indicating that *L. monocytogenes* in Australian environments can survive on the surface of the soil for at least some time.

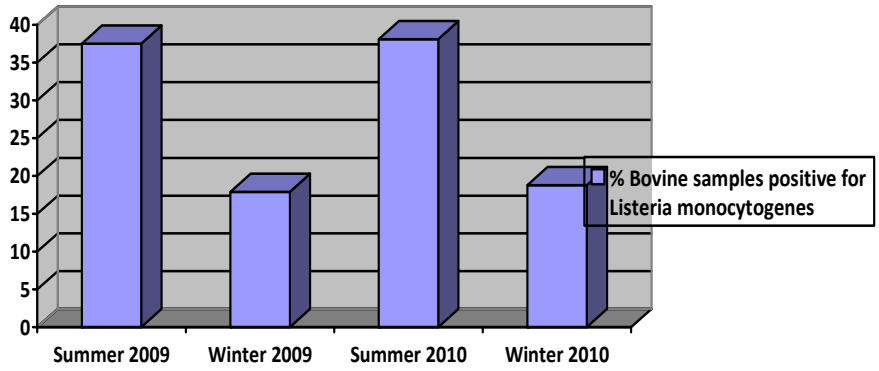
Manure testing

L. monocytogenes has been isolated from faeces of a variety of animal species, including domestic and wild mammals and birds (Seeliger, 1961; Weis and Seeliger, 1975; Yoshida *et al*, 2000). Virtually all species of domestic animals are susceptible to infection by *L. monocytogenes* (Low and Donachie, 1997). Most listeriosis cases in North America occur in cattle (82%), with a smaller percentage in sheep (17%) and pigs (Wesley, 1999). Based on the literature, listeriosis seems to be the biggest problem in domestic ruminants (cattle, sheep and goats) with silage feeding as an important risk factor (Fenlon 1986a; Fenlon *et al.,* 1996; Wiedmann *et al.,* 1996). In addition to relatively small numbers of acutely infected sheep, goats and cattle, substantially more animals within a herd may be asymptomatic carriers (Wesley, 1999). Asymptomatic carriage has also been reported in poultry (review by Wesley, 1999). The results of this study suggest that sheep and cattle are shedders of *L.monocytogenes* in large numbers. This risk factor has been well recognized in Australian risk documents and overall the risk is not because the animal is infected but because the animal is known to be the carrier of this bacterium without actually getting infected.

Ovine manure testing:



Bovine manure testing:



Overall these results support the published evidence that ruminants are heavy shedders of *L. monocytogenes*. Discussions with DPI veterinary staff however indicate that in Australia Listeriosis is a rare disease of animals and certainly Listeriosis is not present in an average of 35% of Australian sheep and 28% of cattle. The results then suggest that ruminants shed this bacterium but are not infected with it. Shedding is usually as a result of ingestion of the bacteria through the feed. A number of reports discuss that feed for ruminants can be contaminated with *L. monocytogenes* (The Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2008)

The association between feed and cattle excretion of *L. monocytogenes* in faeces has been reported. In a study by Fenlon *et al*. (1996), 29–31% of cattle started to shed *L. monocytogenes* after silage feeding. The same serotype and electrophoretic type was found in silage and in faeces of cows fed by that silage, and the level of *L. monocytogenes* in silage ranged from 0.9 to more than 1.1 x 10^6 CFU/g, indicating that the cows were being contaminated with the same type of *L. monocytogenes* found in the feed. Considering that a cow consumes about 40 kg of feed a day, reported numbers of *L. monocytogenes* in silage are likely to result in a significant intake of *L. monocytogenes*.

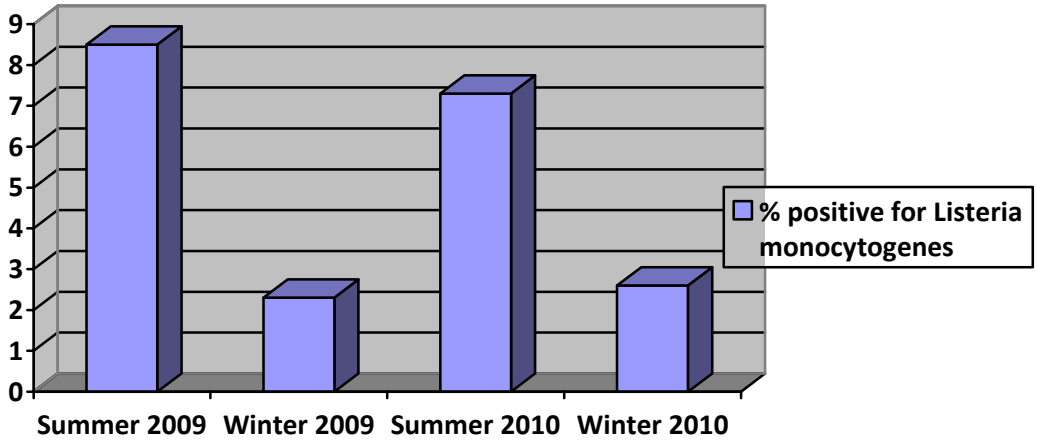
The limitations of the current study are that the manure was collected at random, often samples of manure were collected from a paddock and the possibility exists that the same

animal was involved in a number of samples. The percentage detection however is not that much different from the 29-31% reported in the literature.

Produce testing:

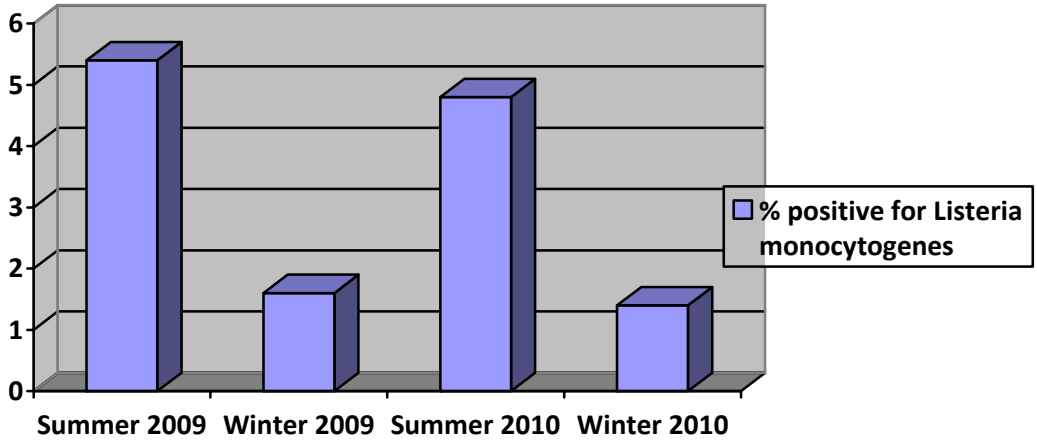
L. monocytogenes has been known to be a contaminant of vegetation. In a study by Weis and Seelinger (1975) in Germany it was shown that *L. monocytogenes* could be detected in 9.7 to 44% of all plants collected. Corn and grain plants were also found to be contaminated at a rate of 10% and 13% respectively. In this particular study the percentage of samples with detectable levels of *L. monocytogenes* ranged from 2.3% in winter to 8.5% in summer. The levels in summer were statistically higher than the levels in winter for both seasons

The average percentage positives for Victorian samples of produce are shown in the following graph.



The results of this study also show that for samples taken from Queensland the levels ranged from 1.4% in winter to 5.4% in summer

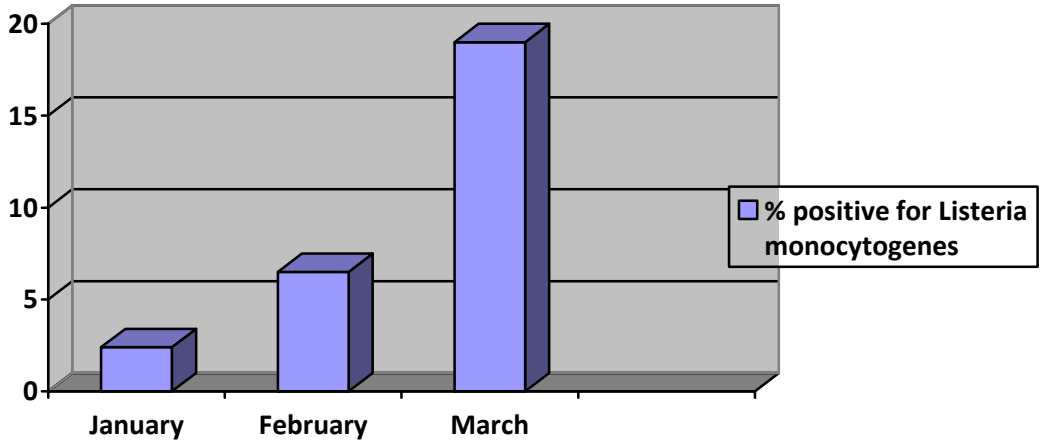
The average percentage positives for Queensland‘s samples of produce is shown in the following graph.



These results suggest that *L. monocytogenes* in produce appears to be more prominent in summer than in winter. No previous results have been published in Australia on the rate of *L. monocytogenes* detection in vegetables. Levels reported in other countries include 22.5% for vegetables in Malaysia (Jeyaletchumi Ponniah *et al* 2010).

The results also suggest that the percentage of contamination is higher in Victoria than in Queensland. Using a binary logistic regression procedure to model the proportion of positive cases, it was found that the odds of a positive case are 3.2 times higher in summer compared to winter ($P<0.001$). We are 95% confident that the true odds ratio is between 1.8 and 6.3. The odds of a positive case are 1.8 times higher in Victoria than Queensland ($P=0.067$). We are 95% confident that the true odds ratio is between 1.0 and 3.5. For these purposes, March was considered part of summer. Note also that there was no data available for January in Queensland, which may have affected these results if January was particularly different to other summer months.

The percentage positive produce samples increased over the months of January to March in Victoria in the summer of the first year of sampling. This increase as shown in the following graph could only be explained by the weather events over that time.



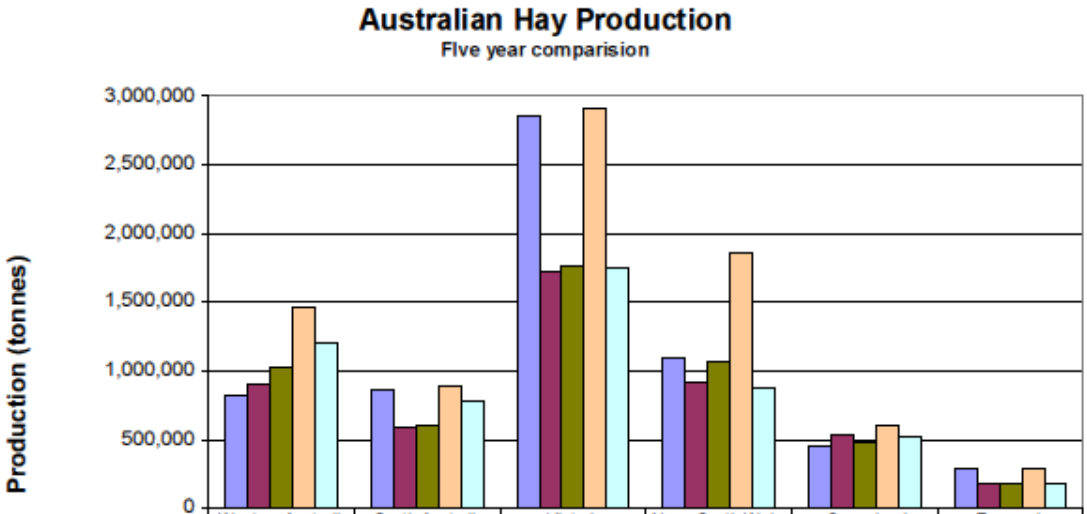
The cause of the increase was thought to be related to the temperature noticed in the weeks preceding the sampling program. Temperatures over this time were extreme, registering 47ºC in some parts of Victoria and Southern NSW. In addition there were two strong wind events over that time. In February, one day registered wind of 83 km/h, in March one day registered 93 km/h. Heat and wind appeared to be the two determining factors that are linked to the increase in positive samples.

Weather events in Victoria in March included a strong (93km/h) Northerly wind on the 3rd of March. A similar SW wind on the 7th of February did not produce as many positives suggesting that the contamination emanated when northerly winds blow on Victorian farms. The contaminating *L. monocytogenes* appears to be carried with dust and particles. The origin of this may well be cow and sheep faeces breaking up in the hot and dry weather and then dispersing with the wind. The theory developed is that animals appear to be infected from

stored bales of hay that may have turned mouldy as suggested by Welshimer & Donker-Volt (1971) and Weis (1975). These stored bales of hay are a source of ingested *L. monocytogenes* for ruminants and then these animals shed the *L. monocytogenes* in their faeces at this time of year. Faeces dry up and end up being dispersed as dust which is carried long distances by strong winds.

No such pattern was noticed in Queensland over the sampling time Queensland was experiencing mild wind conditions (only one day of winds of 50 km/h), in addition to mild rain being prevalent over this time.

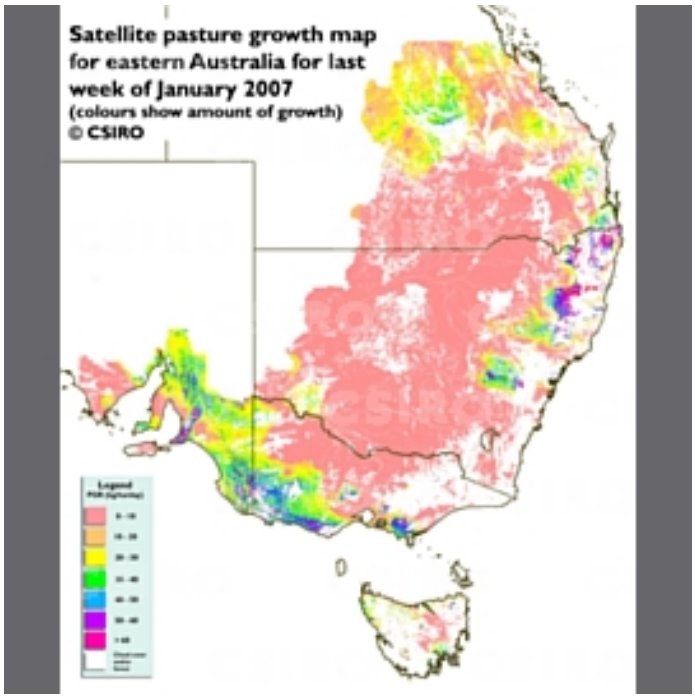
To fully appreciate the theory that baled hay may be the source of dispersed *L.monocytogenes* one needs to understand the dynamics of the grazing industry in Australia. There are 120 M sheep and 28 M cattle in Australia, these animals are supplementary fed hay during our hot dry summers. Overall, Australia is thought to produce 8M metric tons of hay to feed these animals. The hay is baled and stored for up to two years before use. Hay production figures show that Victoria is by far the largest producer of hay.



	Western Australia	South Australia	Victoria	News South Wales	Queesland	Tasmania
2000/01 ABS Census	826,783	863,815	2,852,068	1,100,335	451,693	288,225
2002/03 ABS Survey	905,937	597,133	1,718,397	916,272	543,527	182,578
2006/07 ABS Survey	1,031,108	612,378	1,762,288	1,070,233	482,299	186,013
2007/08 ABS Survey	1,463,249	890,833	2,916,187	1,862,507	609,944	297,727
2008/09 AFIA Est	1,203,847	783,690	1,750,163	877,282	523,569	182,578

Graph showing the figures for Australian hay production, Figures by State, showing high levels of production in the Eastern States. (Hay Supply and demand, Dairy Australia)

The bulk of the grazing areas supplemented by hay are found north of the Victorian sampling sites. Whilst there are substantial grazing sites in Queensland these are not usually supplemented by summer hay feeding as rainfall is usually more common in summer in this area.



Map showing pasture growth for Eastern Australia showing strong pastoral activities to the North of Victoria (CSIRO archives)

Furthermore, the results support the theory that *L. monocytogenes* reservoirs are found in fermented hay. Discussions with veterinary officers from the Department of Primary Industry Victoria suggest that this is not a well known fact and that *L. monocytogenes* infections in Bovines is not an economic issue for that industry. *L. monocytogenes* infections of Ovines on the other hand can lead to sickness in flocks of sheep but the sheep recover quickly and it is not a priority for the industry to tackle this disease at the moment. The fact that hay may be the reservoir on the other hand was of interest to the veterinarians as, until now, they were unsure of where the reservoir was for these bacteria and the assumption was that it was widely spread in the environment but that in Australia no specific origin point was ever noted. There was a time when hay was produced in small bales that were easy to handle, this practice however has switched to very large rolled bales, up to 2 m in diameter, these are left in the field until sold or moved to feed animals. These bales are tightly bound and inside they are anaerobic, causing the content to ferment and produce what is known as silage in the industry. To complicate issues, a large number of bales are produced as protected by plastic and these retain moisture much more effectively than non protected bales.

As a result of climate change silage production in Australia has more than doubled since the early 1990's (http://www.dpi.nsw.gov.au/__data/). ABARE statistics for the dairy industry show that average silage production per dairy farm rose from 64 tons in 1991/92 to 170 tons in 2004/05. Hay production over the same period did not keep pace, rising from 97 tons per dairy farm to 142 tones. ABS statistics show that in 2006 (a drought year) 708,000 t of silage and 1,069,000 t of hay were made in NSW. Silage and hay production across Australia were 2,857,000 t and 5,155,000 t respectively. Silage provides an opportunity to store high quality forage that can maintain high levels of animal production, increase enterprise flexibility and create new marketing opportunities. The need for producers to increase productivity and reduce costs has been a driving force behind the increased use of silage. On many farms, silage making is now a regular annual operation. Prolonged drought and concerns about

climate change are causing other producers to consider the advantages of silage for longer term storage.

Silage is in effect fermented high moisture forage made from plants including hay. It undergoes rapid anaerobic lactic acid fermentation that converts sugars to acids and exhausts any oxygen present in the crop material. The process of fermentation causes a rapid drop in pH and well-preserved silages generally have a pH below 4.5 (Fenlon and Shepherd, 2000). These acidic conditions inhibit the growth of both spoilage microorganisms and *Listeria* (Fenlon and Shepherd, 2000). However, if aerobic conditions are introduced, sufficient fermentation to drop the pH <4.5 may not occur, or growth of aerobic organisms such as yeast and moulds may be initiated, raising the pH of the silage to a point where it provides a favourable environment for the growth of *L.monocytogenes* (Fenlon and Shepherd, 2000). If *L. monocytogenes* is present on the crop due to contamination with dust and small numbers survive during fermentation, *L. monocytogenes* will multiply. Kelly *et al.* (2000) proposed that, as oxygen moves through the silage, the levels of *L.monocytogenes* change for different parts of the whole silage volume and vary throughout the period of silage storage. This is in agreement with Fenlon (1986) who proposed that *Listeria* is found in a microaerophilic ecological niche between totally anaerobic and totally aerobic silage.

It has been reported that *L. monocytogenes* is more often a problem in baled silage than in silo silage (Fenlon, 1985). This is explained by the greater surface area of baled silage exposed to possible aerobic deterioration (Fenlon and Shepherd, 2000). *L. monocytogenes* was isolated from 25.9% of samples of big bale silage and, when mouldy samples were selected, 44% were found to be contaminated (Fenlon, 1985). If silage is contaminated, *L. monocytogenes* can survive for a long period of time. For example, Dijkstra (1971) reported that *L. monocytogenes* can survive 4–6 years in naturally contaminated silage. *Listeria* exposed to sunlight and normal weather however has been shown to die rapidly on vegetation (Appendix3).

Samples from deep inside plastic covered bales show that a large percentage contains *L. monocytogenes* in large numbers (all over 100 CFU/g). This situation is not too different from what happens in cold climates, such as Europe and North America, where animals are supplementary fed in winter when green grass is not available, except that in Australia hay is fed mostly in summer when green grass is not available. This situation has been compounded by climate change, where more hay is fed in summer due to low rain falls.

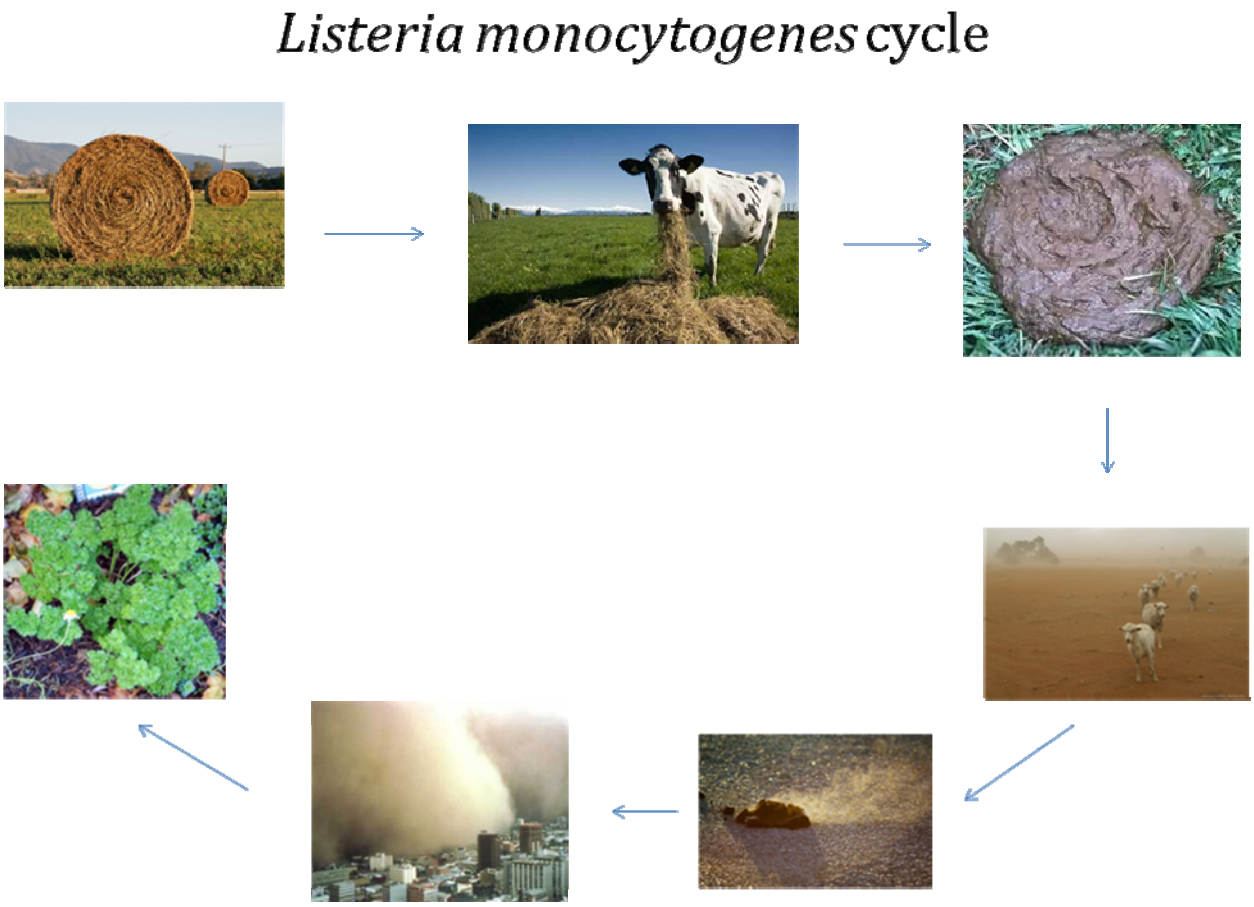
Photographs of some of the bales of hay that tested positive for *L. monocytogenes.* (Photos taken by author)

The concept that *L. monocytogenes* originates in fermented hay and silage is not new, there are many publications that talk about this very subject. Listeriosis in cattle in the Northern hemisphere is often blamed on contaminated feed. Studies by Fendon *et. al.* (1996) show that cows fed on grass shed no *L.monocytogenes* but that almost all the cows fed on silage and fermented bales showed detectable levels of *Listeria spp* including *L. monocytogenes* in their faeces. Studies by Wiedmann *et. al.* (1997) showed that cases of Listeriosis in sheep could be attributed to silage through fingerprinting techniques that linked silage transport equipment with the *L. monocytogenes* strains affecting the sheep. Torriani and Pallotta (1994) recognised the fact that silage was a source of *L. monocytogenes* in silage and developed a rapid test for this microorganism in silage.

The transfer of *L. monocytogenes* from fermented hay to vegetable farms probably occurs through the passing of live *Listeria* in the faeces of ruminants. The faeces dry up in hot dry weather and then they are disrupted by winds and the dust particles generated can travel for many kilometres.

The concept that dust is a carrier of microbial contamination is well documented, although up to now there was no direct scientific evidence that dust was responsible for the spread of *Listeria* to leafy vegetables and herb farms. The potential for survival of *Salmonella* in dust has been reported to be 26 months (Davies and Wray, 1996) and the survival of *E. coli* to be 10 months (Varma *et al,* 2003). The survival of *Listeria* in dust has not been documented but

Listeria generally survives longer than gram negative enteric bacteria like *Salmonella* and *E.coli*. The potential for dust to travel long distances is well documented (up to thousands of km) (Griffin *et al*. 2001). Based on this information a *L. monocytogenes* vegetable contamination cycle has been proposed



There has been a dogma for many years now suggesting that *L. monocytogenes* is a common environmental contaminant but just how this contamination occurred has never been understood. This study suggests for the first time that in Australia *L. monocytogenes* is spread predominantly by the wind on hot dry days. The study supports the circumstantial observation that *Listeria* problems in the leafy vegetable industry are more of an issue in Victoria than in Queensland (other States have not been included in this study and it would be interesting to record the incidence of *L. monocytogenes* in produce from other States).

Hot summer weather followed by strong Northerly winds are a problem for Southern vegetable growers, in winter the threat of *L. monocytogenes* is not completely eliminated but it is reduced as winds in Victoria are mostly from the South. The wet weather would also eliminate dust from the air, thus reducing the spread of *L. monocytogenes*. Contrary to belief, *L. monocytogenes* does not survive for long on healthy plants.

10. Development of best practices for the management of *Listeria monocytogenes* on farms:

"Understanding *Listeria monocytogenes* and vegetable farming"

Food safety has now become entrenched in vegetable growing in Australia; the number of growers that have a QA based food safety system is large and this is now almost mandatory as a condition to supply. Food safety is an issue that will not diminish in importance and is a foundation stone on which the vegetable industry in Australia is built.

One of the microorganisms of importance is *L. monocytogenes,* this bacterium is problematic because it can cause severe illness to consumers and growers must, as part of supply agreements, test for this bacterium regularly. There are many questions related to *L. monocytogenes* that we have not been able to answer. The reason for this is that we have not really studied how this microbe enters vegetable farms in Australia. As a result of a Horticulture Australia funded project we are now in a position to offer some advice to growers. The project carried out testing of soil, manures, water and produce over two years in summer and in winter in growing areas in Queensland and Victoria and has come up with a number of suggestions for growers.

Which test do I specify when testing my produce?

Although there are rapid tests out in the market place, there is no real advantage for growers to use rapid tests as the only legal tests are those based on Australian standards or equivalent. A grower should specify tests that only detect *L. monocytogenes* and that are based on Australian standards or equivalent. Positive tests must be followed by full enumeration. The test must be reported back as negative or positive with enumeration, only then can the grower make decisions about his produce status.

What do I test for to satisfy QA plans that I have low levels of *L. monocytogenes* on my farm?

No point testing soil or water for the presence of *L. monocytogenes. L. monocytogenes* was found to be present in low levels in some water samples, particularly channel water and river water but the levels detected are low and have little or no impact on produce contamination.

What farming practices may contribute directly to *L. monocytogenes* contamination of produce?

L. monocytogenes can multiply in a number of environments over a range of temperatures from low to high (as low as 2ºC). It is important to understand that it needs nutrients and sufficient water to multiply at these temperatures. It multiplies readily in decaying vegetable matter and hence it is important to remove as much of this from the field as possible after harvest. Good field hygiene practices should be followed and green waste should be collected and properly composted at high temperature to destroy any *L. monocytogenes* that may be present. In addition it is important not to use hay or silage in the field operations, particularly stored hay emanating from stored bales.

Do I allow farm animals to enter a vegetable field?

The reality is that cows and sheep do pass *L. monocytogenes* through their digestive system. The *L. monocytogenes* originates from fermented vegetation, usually baled hay/silage. We do not understand how long the animals continue to pass this bacterium after going from hay to fresh grass, so until we better understand this, the best practice is to keep ruminants away from vegetable farms.

What can I do to reduce *L. monocytogenes* from contaminating my produce?

Because *L. monocytogenes* is thought to enter vegetable farms as dust particles emanating from animal faeces in distant sites and carried by strong winds, it is important to institute good practices on the farm that take strong winds in summer into account. It is important to try not to water vegetables just before strong winds (greater than 45Km/h) are expected on hot dry days (wet plants attract more dust). The direction of the winds is also important. In Victoria winds from the north are more likely to carry dust from distant grazing areas. In Queensland, winds from the South are more likely to carry dust from grazing areas.

Preferably water vegetables immediately after the winds subside to wash away any dust.

The study also found that vegetables with lots of leaf curls and lobes attract more dust (and more *L. monocytogenes*) than vegetables that have smooth leaves. Try and select varieties that have fewer curls and lobes.

We know that on healthy plants *L. monocytogenes* dies rather fast (Appendix 3), so try and harvest at least 48 hours after a strong wind event on hot dry days.

There are two other practices that need to be reviewed when it comes to *L. monocytogenes* in vegetable farms. Rotting vegetation is considered as primary source for *L.monocytogenes* so field hygiene is important. Do not dig in waste vegetation unless it is properly composted (composting increases the temperature and kills *L. monocytogenes*). In addition, the practice of digging in green manure needs to be evaluated as a possible source of *L.monocytogenes* growth. Most importantly, do not use hay as part of your farming practices unless the hay is fresh and has not undergone fermentative breakdown.

How do I remove *L. monocytogenes* from my produce if I suspect that it has been exposed to dust on hot dry days?

Washing of vegetables before delivering to a market is still the best way to reduce the level of *L. monocytogenes* on the produce. It is important however, to use a turbulent washing system as static washing systems do very little in reducing the level of dust on produce. The best way is to use a turbulent wash bath containing an AVPMA approved sanitiser capable of killing bacteria in solution.

11. Technology Transfer

A range of methods of technology transfer have been used as part of this project, the most important was direct discussions with a number of growers that have experienced issues

related to *L. monocytogenes* detections in their produce. An information sheet has been published and distributed as requested. Furthermore some of the results from this study have been presented at the Victorian Vegetable Grower Association 2009 annual meeting. Some results were presented at the 2009 Food Science and Technology conference in Melbourne (February 2009). The results were also presented at the South East Asian Fresh Produce Quality conference in August 2010. It is hoped that the information gathered as part of this project will be presented as a Veg note on microbial contaminants in the vegetable industry. A number of articles have been produced related to this project. The results of this project will be used to further strengthen on farm QA systems, including Freshcare and Salad GAP.

12. Project Evaluation

The information supplied to growers has led to a better understanding of *L. monocytogenes,* some growers are asking about washing systems and testing procedures and are reading the information presented in various formats. A full evaluation of the effectiveness of this project however should be carried out in 2 years from the completion date to see how the information supplied will be used by growers to change their practices and to see if it has contributed to a reduction in detections of *L. monocytogenes* in the leafy vegetable industry.

13. Conclusions

• Water is not a major vehicle for *L. monocytogenes* contamination of leafy vegetables

• Chicken manure is not a predominant means of *L. monocytogenes* contamination of leafy vegetables

• Marsupial faeces is not a predominant means of *L. monocytogenes* contamination of leafy vegetables

• Cow and sheep manure can be heavily contaminated with *L. monocytogenes* so that direct contact of leafy vegetables growing areas should be avoided especially if the animals have been fed on silage or hay recently

• Contamination of produce in summer appears to be higher than in winter

• Contamination appears to be higher after very hot days and wind from animal grazing sites

• A *L. monocytogenes* cycle has been proposed, this involves *L. monocytogenes* present inside baled hay passing through the faeces of a cow or a sheep, upon drying the dust from the faeces is dispersed by wind, high wind speeds could contaminate distant places

• Listeria spp seem to die off rapidly in healthy plants

• A leaflet has been prepared to assist vegetable growers dealing with this contaminant

• Testing for *L. monocytogenes* must be specified when sending samples to laboratories for analysis

• Rapid tests based on virulence testing may be more helpful for the industry as they become available in the future.

14. Key Issues

• Dust spread by winds after hot days seems to be the major cause of *L. monocytogenes* contamination of vegetable farms.
• Rapid tests may not necessarily be beneficial for the vegetable industry, tests based on Australian standards on the other hand should be seen by the industry as the only tests to use to comply with due diligence expectations, even if these tests take longer.
• In the future, better tests that can discriminate between virulent and non virulent *L. monocytogenes* may be of assistance to the industry.
• Turbulent washing systems, combined by a suitable sanitiser, may be useful in reducing or eliminating *L. monocytogenes* from produce, static washing may not remove all traces of dust on the produce.
• Field hygiene principles may be important in reducing *L. monocytogenes* contamination on farm and the use of old hay or uncomposted vegetable matter should be eliminated.

15. Recommendations for Future Work

The recommendations for future work are as follows

- Testing other areas of Australia where leafy vegetables are grown, such as southern Tasmania and Southern areas in Western Australia to better understand how dust plays a part in contamination of leafy vegetables.

- *L. monocytogenes* contains a number of antigens; these have been used to make specific antisera that can be used to detect serotypes of *L. monocytogenes* by agglutination. This in turn can separate *L. monocytogenes* strains into at least 12 different serotypes (i.e., 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, and 7). What is interesting is that serotypes 1/2a, 1/2c, 1/2b, and 4b account for over 98% of the reported human listeriosis cases. Hence a rapid test that discriminates these serotypes from the other serotypes would be extremely beneficial for the horticultural industry. It may lead to virulent and infective *L. monocytogenes* being considered a rare contaminant of leafy vegetables.

16. References

Arnold, G. J. and Coble. J. (1995). Incidence of *Listeria* species in foods in NSW.

Food Australia 47 (2): 71-75.

Australian Associated Press (1999). Coroner suspects six may have died from fruit salad. September 8.

Beuchat LR (1996). Pathogenic microorganisms associated with fresh produce. J. Food Prot., 59:204-216.

Crerar, S.K., Dalton, C.B., Longbottom, H.M. and Kraa, E. (1996). Food borne disease: current trends and future surveillance needs in Australia. MJA, 165: 672-675.

Cordano A.M. and Jacquet C. (2009) *Listeria monocytogenes* isolated from vegetable salads sold at supermarkets in Santiago, Chile: Prevalence and strain characterization. International Journal of Food Microbiology 132: 176–179

Davies, RH and Wray, C. (1996). Persistence of Salmonella enteritidis in poultry units and poultry food. British Poultry Science, 37, 589-596.

de Simon, M., Tarrago, C. and Ferrer. M.D. (1992). Incidence of *Listeria*

monocytogenes in fresh foods in Barcelona (Spain). Int. J. Food Microbiol. 16:153-156.

Dijkstra RG. (1982). The occurrence of *Listeria monocytogenes* in surface waters of canals and lakes, in ditches of one big polder and in the effluents of canals of a sewage treatment plant. Zentralbl Bacteriol 176:202–205.

Dijkstra RG (1971). Investigations on the survival times of *Listeria* bacteria in suspensions of brain tissue, silage and faeces and in milk. Zentralbl Bacteriol 216:92–95.

Fenlon DR. (1985). Wild birds and silage as reservoirs of *Listeria* in the agricultural environment. J Appl Bacteriol 59: 537–543.

Fenlon DR. (1986). Rapid quantitative assessment of the distribution of *Listeria* in silage implicated in a suspected outbreak of listeriosis in calves. Vet Rec 118:240–242.

Fenlon, D. R. (1999). *Listeria monocytogenes* in the natural environment, p. 21-38. *In* E. T. Ryser and E. H. Marth (ed.), *Listeria*, listeriosis, and food safety, 2nd ed. Marcel Dekker, New York, N.Y.

Fenlon DR, Wilson J, Donachie W.(1996). The incidence and level of *Listeria monocytogenes* contamination of food sources at primary production and initial processing. J Appl Bacteriol 81:641–650.

Fenlon DR, Shepherd JL. (2000). Ecology of *Listeria monocytogenes*: Studies on Incidence, Growth, and Microbial Competition in Primary Production. Proceedings from the ILSI North America Symposium Series on Food Microbiology; 26–28.

Gola, S., Previdi, M.P., Mutti, P. and Bellodi, S. (1990). Microbiological investigation of frozen vegetables: incidence of *Listeria* spp. and other psychrophillic pathogens.

Industria Conserve. 65: 36-38.

Gray ML, Killinger AH. (1966). *Listeria monocytogenes* and listeric infection. Bacteriol Rev 30:308–382.

Griffin, DW; Garrison, VH; Herman, JR and Shinn, EA. (2001). African desert dust in the Caribbean atmosphere: Microbiology and public health. Aerobiologia, 17, 203–213.

Gouws P.A. and I. Liedemann (2005). Detection of *L. monocytogenes* in Food Products, *Food Technol. Biotechnol. 43* (2) 201–205

Hay supply and demand report.
http://www.ntaga.org.au/_assets/0909_HaySupplyEstimateReport.pdf

Jeyaletchumi Ponniah, , Tunung Robin, Margaret Selina Paie, Son Radu, Farinazleen Mohammad Ghazali, Cheah Yoke Kqueen, Mitsuaki Nishibuchi, Yoshitsugu Nakaguchi and Pradeep Kumar Malakar (2010). *Listeria monocytogenes* in raw salad vegetables sold at retail level in Malaysia. Food Control Volume 21, Issue 5, May 2010, Pages 774-778

Jon I Esteban, Beatriz Oporto, Gorka Aduriz, Ramón A Juste and Ana Hurtado (2009). "Faecal shedding and strain diversity of *Listeria monocytogenes* in healthy ruminants and swine in Northern Spain" *BMC Veterinary Research*, 5:2

Heisick, J. E., Wagner, D. E., Nierman, M. L. and Peeler, J. T. (1989) *Listeria* spp. found on fresh market produce. *Appl. Environ. Microbiol.* 55, 1925–1927.

Kelly LA, Gibson G, Gettinby G, Donachie W, Low JL (2000). A predictive model of the extent of listerial contamination within damaged silage bales. Quant Microbiol 2: 171–188.

Low JC, Donachie W. (1997). A review of *Listeria monocytogenes* and listeriosis. Vet J 153:9–29.

Schlech, W.F., Lavigne, P.M., Bortulussi, R.A., Allen, A.C., Haldane. E.Y. Worth,

A.J., Hightower, A.W., Johnson, S.E., King, S.H., Nicholls. E.S. and Broome. C.V.

(1983). Epidemic listeriosis- evidence for transmission by food. N. Eng. J. Med. 308:

303-206.

Seeliger HPR. Listeriosis 2nd edition. Basel: Karger, 1961

Sizmur, K. and Walker, C.W. (1988). *Listeria* in prepacked salads. The Lancet. (May

21): 1167.

The Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Terrestrial Manual) 2008.

http://www.cabi.org/ahpc/default.aspx?site=160&page=3323

Torriani S. and M. L. Pallotta (1994). Use of Polymerase Chain Reaction to detect Listeria monocytogenes in silages. Biotechnology Techniques 1994 Volume 8, Number 3, 157-160

Varma, JK; Greene, KD; Reller, ME; DeLong, SM; Trottier, J; Nowicki, SF; DiOrio, M; Koch, EM; Bannerman, TL; York, ST; Lambert-Fair, M-A.; Wells, JG and Mead, PS. (2003). An outbreak of Escherichia coli O157 infection following exposure to a contaminated building. The Journal of American Medical Association, 290, 2709-2712.

Wesley IV. Listeriosis in Animals. In Ryser ET (ed.): *Listeria*, Listeriosis, and Food Safety. New York: Dekker, 1999:39–73.

Weis, J. 1975. The incidence of *Listeria monocytogenes* on plants and in soil. In *Problems of Listeriosis.* Proceedings of the 6th International Symposium on Listeriosis ed. Woodbine, W. pp. 61-63 Leicester: Leicester University Press.

Weis J, Seeliger HPR. (1975) Incidence of *Listeria monocytogenes* in nature. Appl Microbiol 30:29–32.

Welshimerh j. and Donker-Voitj. (1971). *Listeria monocytogenes* in nature. *Applied Microbiology* 21, 516-519.

Wesley IV. Listeriosis in Animals. In Ryser ET (ed.): *Listeria*, Listeriosis, and Food Safety. New York: Dekker, 1999:39–73.

Wiedmann M., Arvik T., Bruce J.L., Neubauer J., Del Piero F., Smith M.C., Hurley J,, Mohammed H.O. and Batt C.A. (1997). Investigation of a listeriosis epizootic in sheep in New York state. *Am. J. Vet. Res*., 58, 733–737.

Wiedmann, M., J. Czajka, N. Bsat, M. Bodis, M. C. Smith, T. J. Divers, and

C. A. Batt. (1994). Diagnosis and epidemiological association of *Listeria monocytogenes*

strains in two outbreaks of listerial encephalitis in small ruminants.

J. Clin. Microbiol. 32:991–996.

Wong, H.C, Chao, W.L, Lee, S.J. (1990). Incidence and characterisation of *Listeria*

monocytogenes in foods available in Taiwan. App and Env Micro. 56: 3101-3104.

http://www.dpi.nsw.gov.au/__data/assets/pdf_file/0005/229595/Silage-Note-1---Role-for-silage-in-Australian-grazing-systems.pdf

Yoshida T, Sugimoto T, Sato M, Hirai K. (2000) Incidence of *Listeria monocytogenes* in Wild Animals in Japan. J Vet Med Sci 62:673–675.

Appendix 1: Results obtained with the three *L. monocytogenes* tests used

Sample N°	*Listeria monocytogenes* status	Australian standard method for *Listeria monocytogenes* testing	Vidas	PCR
1	+ve *Listeria monocytogenes*	+ve	+ve	+ve
2	+ve *Listeria monocytogenes*	+ve	+ve	+ve
3	+ve *Listeria monocytogenes*	+ve	+ve	+ve
4	+ve *Listeria monocytogenes*	+ve	+ve	+ve
5	+ve *Listeria monocytogenes*	+ve	+ve	+ve
6	+ve *Listeria monocytogenes*	+ve	+ve	+ve
7	+ve *Listeria monocytogenes*	+ve	+ve	+ve
8	+ve *Listeria monocytogenes*	+ve	+ve	+ve
9	+ve *Listeria monocytogenes*	+ve	-ve	+ve
10	+ve *Listeria monocytogenes*	+ve	+ve	+ve
11	+ve *Listeria monocytogenes*	+ve	+ve	+ve
12	+ve *Listeria monocytogenes*	+ve	+ve	+ve
13	+ve *Listeria monocytogenes*	+ve	+ve	+ve
14	+ve *Listeria monocytogenes*	+ve	+ve	+ve
15	+ve *Listeria monocytogenes*	+ve	+ve	+ve
16	+ve *Listeria monocytogenes*	+ve	+ve	+ve
17	+ve *Listeria monocytogenes*	+ve	+ve	+ve
18	+ve *Listeria monocytogenes*	+ve	+ve	+ve
19	+ve *Listeria monocytogenes*	+ve	+ve	+ve
20	+ve *Listeria monocytogenes*	+ve	+ve	+ve
21	+ve *Listeria monocytogenes*	+ve	+ve	+ve
22	+ve *Listeria monocytogenes*	+ve	+ve	+ve
23	+ve *Listeria monocytogenes*	+ve	+ve	+ve

24	+ve *Listeria monocytogenes*	+ve	+ve	+ve
25	+ve *Listeria monocytogenes*	+ve	**-ve**	+ve
25	+ve *Listeria monocytogenes*	+ve	+ve	+ve
27	+ve *Listeria monocytogenes*	+ve	+ve	+ve
28	+ve *Listeria monocytogenes*	+ve	+ve	+ve
29	+ve *Listeria monocytogenes*	+ve	+ve	+ve
30	+ve *Listeria monocytogenes*	+ve	+ve	+ve
31	+ve Listeria spp.	-ve	-ve	-ve
32	+ve Listeria spp.	-ve	-ve	-ve
33	+ve Listeria spp.	-ve	**+ve**	-ve
34	+ve Listeria spp.	-ve	-ve	-ve
35	+ve Listeria spp.	-ve	-ve	-ve
36	+ve Listeria spp.	-ve	-ve	**+ve**
37	+ve Listeria spp.	-ve	-ve	-ve
38	+ve Listeria spp.	-ve	-ve	**+ve**
39	+ve Listeria spp.	-ve	-ve	-ve
40	+ve Listeria spp.	-ve	-ve	-ve
41	+ve Listeria spp.	-ve	-ve	-ve
42	+ve Listeria spp.	-ve	-ve	-ve
43	+ve Listeria spp.	-ve	**+ve**	-ve
44	+ve Listeria spp.	-ve	-ve	-ve
45	+ve Listeria spp.	-ve	-ve	-ve
46	+ve Listeria spp.	-ve	-ve	-ve
47	+ve Listeria spp.	-ve	-ve	-ve
48	+ve Listeria spp.	-ve	**+ve**	-ve
49	+ve Listeria spp.	-ve	-ve	**+ve**
50	+ve Listeria spp.	-ve	-ve	-ve

51	-ve	-ve	-ve	-ve
52	-ve	-ve	-ve	-ve
53	-ve	-ve	**+ve**	-ve
54	-ve	-ve	-ve	-ve
55	-ve	-ve	-ve	-ve
56	-ve	-ve	**+ve**	+ve
57	-ve	-ve	-ve	-ve
58	-ve	-ve	-ve	**+ve**
59	-ve	-ve	-ve	-ve
60	-ve	-ve	-ve	-ve

Appendix 2: Results of water testing for *L. monocytogenes*

Sample N^{o}	Type	L. monocytogenes detection/100ml Season 1 (Summer)	L. monocytogenes detection/100ml Season 1 (Winter)	L.monocytogenes detection/100ml Season 2 (Summer)	L. monocytogenes detection/100ml Season 2 (Winter)
1	Dam	-ve	-ve	ND	ND
2	Dam	+ve	-ve	-ve	-ve
3	Dam	-ve	-ve	-ve	+ve
4	Dam	-ve	-ve	-ve	-ve
5	Dam	-ve	-ve	-ve	-ve
6	Irrigation pipe	-ve	ND	ND	ND
7	Irrigation pipe	-ve	ND	ND	ND
8	Dam	-ve	-ve	-ve	-ve
9	Dam	-ve	-ve	-ve	-ve
10	Dam	-ve	-ve	-ve	-ve
11	River	-ve	-ve	-ve	-ve
12	River	-ve	-ve	-ve	-ve
13	Dam	-ve	-ve	-ve	ND
14	Dam	-ve	-ve	-ve	-ve
15	Dam	-ve	-ve	-ve	-ve
16	Irrigation channel	-ve	+ve	-ve	-ve
17	Irrigation channel	+ve	+ve	-ve	+ve
18	Dam	-ve	-ve	-ve	ND
19	Dam	-ve	-ve	-ve	-ve
20	Lake	-ve	-ve	-ve	-ve
21	Lake	-ve	-ve	-ve	-ve
22	Dam	-ve	-ve	-ve	-ve
23	Irrigation pipe	-ve	-ve	-ve	ND
24	Irrigation pipe	-ve	-ve	-ve	ND
25	Bore water	-ve	-ve	-ve	-ve
25	Bore water	-ve	-ve	-ve	-ve

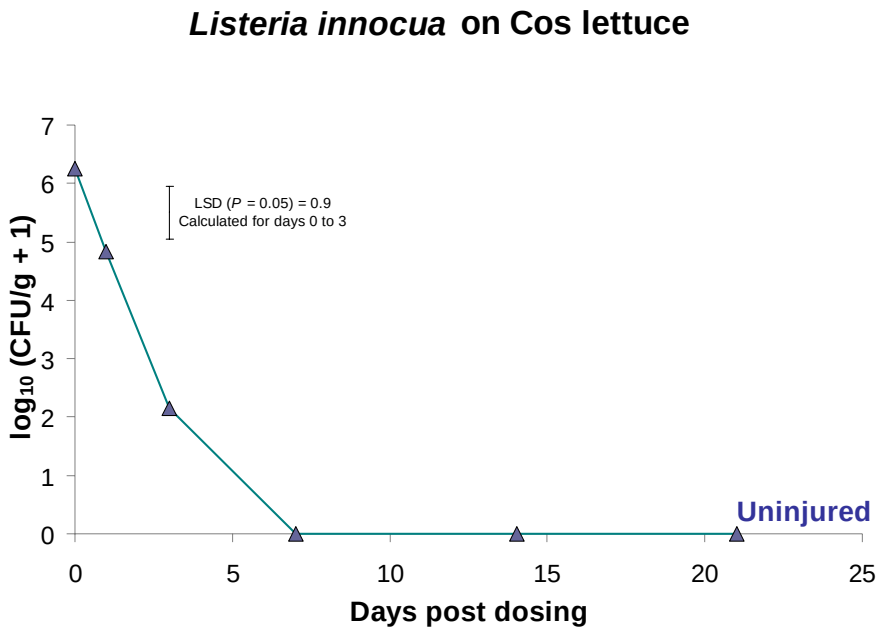
27	Bore water	-ve	-ve	ND	ND
28	Dam	+ve	+ve	-ve	-ve
29	Dam	-ve	-ve	-ve	-ve
30	Dam	-ve	-ve	-ve	-ve
31	Recycled water	-ve	-ve	-ve	-ve
32	Dam	-ve	-ve	-ve	-ve
33	Dam	-ve	-ve	-ve	-ve
34	Dam	-ve	-ve	-ve	-ve
35	Bore water	-ve	-ve	-ve	-ve
36	Dam	-ve	-ve	-ve	ND
37	River	-ve	-ve	+ve	-ve
38	River	+ve	-ve	-ve	-ve
39	Dam	-ve	-ve	-ve	-ve
40	Dam	-ve	-ve	-ve	-ve
41	Dam	-ve	-ve	-ve	-ve
42	Dam	-ve	-ve	-ve	-ve
43	Pond	-ve	ND	-ve	-ve
44	Pond	-ve	ND	-ve	-ve
45	River	-ve	-ve	+ve	-ve
46	River	+ve	-ve	ND	-ve
47	Irrigation pipe	-ve	ND	-ve	-ve
48	Irrigation pipe	-ve	ND	-ve	-ve
49	Dam	-ve	-ve	-ve	-ve
50	Dam	-ve	-ve	-ve	+ve
51	Dam	-ve	-ve	-ve	-ve
52	Dam	-ve	-ve	-ve	-ve
53	Irrigation pipe	-ve	ND	-ve	-ve
54	Irrigation pipe	-ve	ND	-ve	-ve
55	Irrigation channel	-ve	ND	-ve	ND
56	Irrigation channel	-ve	ND	+ve	ND

57	Dam	-ve	-ve	ND	-ve
58	Dam	-ve	-ve	-ve	-ve
59	Irrigation pipe	-ve	ND	-ve	-ve
60	Irrigation pipe	-ve	ND	-ve	-ve
61	Irrigation pipe	-ve	ND	-ve	-ve
62	River	-ve	-ve	-ve	-ve
63	River	-ve	-ve	-ve	-ve
64	Irrigation pipe	-ve	ND	-ve	-ve
65	Irrigation pipe	-ve	ND	-ve	-ve
66	Small holding dam	-ve	-ve	ND	-ve
67	Dam	-ve	-ve	-ve	-ve
68	Dam	-ve	-ve	ND	-ve
69	Dam	-ve	-ve	+ve	-ve
70	Irrigation pipe	-ve	ND	-ve	ND
71	Irrigation pipe	-ve	ND	-ve	ND
72	Irrigation pipe	-ve	-ve	-ve	-ve
72	Irrigation pipe	-ve	-ve	-ve	-ve
73	Dam	-ve	-ve	-ve	-ve

Appendix 3: Survival of *Listeria* on the surface of leafy vegetables

Although *L. monocytogenes* was detected in high levels in moist fermented hay, an experiment was carried out to better understand how long the *Listeria* bacterium could survive for on the surface of a leafy vegetable.

Persistence of *L. innocua* colony forming units (CFU) on the shoots of uninjured glasshouse cos lettuce sprayed with *L. Innocua* at relatively high levels (1 x 10^6 per gram). This was enumerated with *Listeria* Selective Agar (Oxford formulation)... Each point represents the mean of log-transformed (+ 1) counts from 6 plants. Points differing by the least significant difference (LSD) or more are statistically significant. The levels of *Listeria* were then measured over 25 days post contamination.



Results show that levels of *Listeria* decline rapidly on the exposed leaves, falling to four logs within 2 days of inoculation.