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**MSc Dissertation**

# Effect of irrigation intervals and processing on the survival of *Listeria monocytogenes* on spray irrigated broccoli

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**A dissertation submitted in partial fulfillment of the requirements for the degree MSc (Food Science)**

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# DECLARATION

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Mignon Crous                                                                                Date

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# ABSTRACT

The first aim of this study was to determine the effect of irrigation intervals on the survival of *L. monocytogenes* on spray irrigated broccoli under field trial conditions, and subsequent survival of the pathogen on broccoli during postharvest processing procedures. The nonpathogenic *L. innocua* was used as surrogate organism to *L. monocytogenes*.

Broccoli in the field was treated with irrigation water inoculated with *L. innocua*, during intervals over a period of five weeks and the growth and survival of the organism was monitored weekly. *L. innocua* numbers remained similar over intervals that received consecutive inoculations and *L. innocua* numbers decreased by at least 2.3 log cfu/g after inoculation ceased, which showed an inoculation effect and that time had an influence on organism survival. Cessation of irrigation before harvest was found to effectively reduce pathogen contamination levels on the crop, whilst repeated irrigation with contaminated water contributes to maintenance of *L. innocua* as well as elevated total microbial counts on the broccoli. A lack of correlation between the *L. innocua* counts and the recorded environmental temperatures in the field, including temperature and relative humidity, suggested that survival is not solely dependent on and influenced by, nor can it be predicted by these parameters. It was found that the presence of high levels of contamination (with, in this case *L. innocua*) in irrigation water used for vegetable crops, can be associated with an increased microbial population on the crop surface.

Secondly, the effect of processing on organism survival post-harvest was assessed. Washing with water caused a 1 log reduction of *L. innocua*, whilst washing with 200 ppm chlorinated water facilitated a further 1 log reduction. Cooking reduced *L. innocua* numbers on broccoli by an average of 1.1 log units and aerobic plate counts by between 1 and 2 log units. A combined treatment of washing with chlorine, storage in MAP (5% $CO_2$, 5% $O_2$) for two days at 4°C and final microwave heating resulted in the lowest pathogen numbers, causing a 5.13 log cfu/g log reduction. Therefore, even though chlorine is effective in reducing *L. innocua* during minimal processing, it does not suffice alone to eliminate pathogens (with *L. innocua* being representative of *L. monocytogenes*) from vegetables, just as MAP storage is only effective as part of a hurdle procedure. Cooking is essential in destroying *L. innocua*

present on broccoli and to ensure vegetables that are safe for consumption in terms of pathogenic exposure.

With this knowledge on the behaviour of *L. monocytogenes* on broccoli, the risk associated with the application of contaminated irrigation water to fresh produce can be better understood and the hazard managed.

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# CHAPTER 1 INTRODUCTION

Fresh produce is a commodity widely traded in South Africa, locally and for export (M. Scheepers, personal communication, 2010). Fresh and minimally processed fruits and vegetables (MPFs) provide a large proportion of our daily nutritional requirements and as healthy eating trends become more popular and consumers become more aware of the benefits of their consumption in reducing the risk of various lifestyle diseases, their desirability increases (Britz, 2005).

In South Africa, fresh produce such as broccoli, a vegetable rich in nutrients, is often sold at fresh produce markets. Farmers deliver their produce to market agents who then sell it directly to the consumer (Anon., 2011a). Such produce therefore undergoes little or no form of processing, providing pathogenic contaminants the opportunity to be transported on the crop, creating the risk of transfer to the consumer (Johnston *et al*., 2005).

The quality of water sources in South Africa have come under the spotlight in previous years. In South Africa, fresh water is scarce and is decreasing in quality because of an increase in pollution and the destruction of river catchments, caused by urbanisation, deforestation, creation of dams in rivers, destruction of wetlands, industrial development, mining, agriculture, energy use, and accidental water pollution through leakage of sewage and effluent into water sources (Anon., 2011b). As population levels increase, the demand for limited water sources increases. There is a growing awareness that good-quality irrigation water is an important factor in the production of safe fruit and vegetables (Steele & Odumeru, 2004).

## 1.1. PROBLEM STATEMENT

During 2007, a study was initiated to determine whether irrigation water, with high levels of bacterial contamination, contributes to the bacterial contamination on vegetable produce in the field. The preliminary results of the study confirmed the presence of pathogens such a *Listeria monocytogenes* and *Escherichia coli* both on vegetables and in irrigation water, thus suggesting a possible relationship between the bacterial water quality and the bacterial safety of the vegetables, including broccoli, lettuce and spinach (Ijabadeniyi & Buys, 2011).

Broccoli harvested from the field was found to contain pathogens, of which *L. monocytogenes* was one (Ijabadeniyi & Buys, 2011). *L. monocytogenes* is a gram-positive, asporogenous organism which is widely distributed throughout the environment and has been isolated from various plant and animal products associated with foodborne illness outbreaks. Ingestion of the organism can cause the disease listeriosis (Pearson & Marth, 1990). *L. monocytogenes* is known to survive under certain adverse conditions, even at temperatures as low as 0.5°C (Peiris, 2005). Berrang *et al*., (1989) reported that populations of *L. monocytogenes* increased during controlled atmosphere storage. The versatility, adaptability and resistance of the pathogen are important factors in minimally processed vegetables as its phsychrotrophic properties render refrigeration temperatures insufficient to ensure the safety of stored food against *L. monocytogenes* (Lee *et al*., 2007).

Broccoli is a winter crop and therefore grows in environmental conditions of relatively low temperature (ideally between 18 and 23°C) and humidity depending on the region, explaining why *L. monocytogenes* is a factor of consideration in the cultivation of this vegetable (Pearson & Marth, 1990). *L. monocytogenes* has been found to rapidly attach to and multiply on plant surfaces and colonise intercellular spaces where it may be protected from sanitation treatments, which poses a problem during processing treatments (Milillo *et al*.,, 2008).

After harvesting, broccoli undergoes minimal processing procedures including washing and packaging under modified atmosphere. Modified atmosphere packaging, in combination with refrigeration, is increasingly being employed as a mild preservation technique to ensure quality and shelf-life, as the demand for fresh, convenient, minimally processed vegetables increases. The fresh nature of these products, together with the mild processing techniques and subsequent storage conditions have presented organisms such as *L. monocytogenes* with new potential infection opportunities and vehicles (Francis *et al*., 1999). Less hurdles exist to eliminate these organisms and conditions for survival are more favourable and of such a nature so as to support organism growth rather than suppressing it. The investigation of the effect of these processing procedures on the growth of *L. monocytogenes* on broccoli is therefore an area of further interest.

As the incidence of pathogens such as *L. monocytogenes* in irrigation water (Lötter, 2010) and on broccoli has been confirmed in various reports (Ijabadeniyi & Buys, 2011; Beuchat, 1996), the question now remains, to what extent the pathogen

present in the irrigation water attaches to the broccoli and whether it survives and proliferates on the broccoli under the environmental conditions in the field. A paper by Crépet *et al*. (2007), showed that surveys conducted after 2000 reported lower instances of *L. monocytogenes* isolation, suggesting that increased knowledge of the behaviour of the pathogen in the food production environment and more effective sanitisation procedures have led to improved product control.

This project aims to provide knowledge on the presence of the pathogen on broccoli. From this, the probability of exposure to the pathogen and the subsequent risk this poses to the consumer upon consumption of the broccoli can be estimated. This study used the surrogate *L. innocua* as a model organism to determine whether *L. monocytogenes* present in irrigation water attaches to broccoli and whether it survives on the broccoli under the reigning environmental conditions, as well as determine the effect of minimal processing on the survival of *L. innocua* present on broccoli.

# CHAPTER 2 LITERATURE REVIEW

## 2.1. WATER CONTAMINATION AND FOOD BORNE DISEASE

### 2.1.1. Irrigation water

Irrigation water sources include groundwater, surface water and human wastewater (Steele & Odumeru, 2004). Groundwater is located in aquifers beneath the earth's surface and surface water includes that of freshwater sources such as lakes, rivers and streams. Wastewater, referring to water containing human sewage, is commonly used for irrigation in countries with limited water sources (Steele & Odumeru, 2004). Irrigation water in South Africa is commonly sourced from large dams and rivers, ground water and industrial reservoirs. Surface waters, including rivers and streams, are very susceptible to contamination with pathogenic microorganisms (Steele & Odumeru, 2004). Polluted irrigation water originates from household waste being discarded into rivers and dams, sewage discharges, storm drains and industrial effluents. Informal settlements have insufficient waste removal systems, resulting in human waste flowing into water catchments irrigation sources and poorly maintained sewerage pipelines or poorly operated wastewater treatment works pollute irrigation water sources (Britz, *et al*., 2007). Irrigation with such poor-quality water is one of the most prominent causes of pathogen contamination of fruit and vegetables, as the review by Steele & Odumeru, (2004) states. In South Africa, farmers utilise about 65% of the nation's fresh water, of which 33% is applied for the irrigation of crops, with sprinkler irrigation mainly being used for vegetable crops. Domestic foodstuffs and a very large percentage of agricultural exports are derived from irrigated lands (DWAF, 1996). Such a high usage of water, together with the fact that the country is in a semi-arid zone with a climate more suited for live-stock grazing than crop production (Zimmerman, 2000), clearly shows the need for the provision of water of high quality (Barnes, 2003).

During 2007, a study was initiated by the Water Research Commission (WRC) of South Africa to determine whether irrigation water, with high levels of bacterial

contamination, contributes to the bacterial contamination on vegetable produce in the field. Several rivers in the Western Cape, Gauteng, Mpumalanga and North West provinces of South Africa were identified as sources of irrigation water for food crops. The water from rivers such as the Berg river, Bree river, Olifants and Wilge rivers, among others, along with the produce from the fields being irrigated with water from these sources was analysed for microbial contamination. After investigation of the Gauteng, Mpumalanga and North-West provinces, results confirmed the presence of pathogens, including *E.coli*, *S.aureus*, *L. monocytogenes* and *Salmonella* in the water and also on the irrigated produce (Ijabadeniyi & Buys, 2011). *L. monocytogenes* specifically, was detected in 37.5%, 37.5% and 62.5% of water samples from the Loskop canal, Olifants and Wilge rivers respectively and on 25% and 75% of cauliflower and broccoli samples respectively, on one of the farms. These figures were similar for produce from all of the investigated sites. These results suggest a possible relationship between the bacterial water quality and the bacterial safety of the vegetables, including broccoli, lettuce and spinach (Ijabadeniyi & Buys, 2011). Lötter (2010), assessed the microbial loads present in rivers used for irrigation of vegetables in the Western Cape, South Africa. They concluded that the rivers investigated during their study contained high levels of contamination and that their results suggest a carry-over of pathogens from the river water to the irrigated produce.

The ability of a pathogen to survive in the environment as well as on fruit and vegetables is an important determinant in the potential risk of disease from the ingestion of produce. Although the viabilities of most pathogens in the environment decrease over time, pathogens have been reported to survive in water and on the surfaces of produce for up to 15 days (Steele & Odumeru, 2004).Furthermore, the method of irrigation has an influence on the extent of contamination of the crop. During spray irrigation the irrigation water comes into contact with the edible portions of the plant, thus posing a higher risk for pathogen transmission (Solomon *et al.*, 2002).

According to Steele & Odumeru (2004), guidelines governing the microbial quality of irrigation water differ greatly between countries and this inconsistency reflects considerable uncertainty about the actual risk of disease transmission related to pathogens present in irrigation water. The quality of water recommended for irrigation of crops likely to be consumed raw is often higher than that for processed crops.

### 2.1.2. Fresh produce and foodborne illness

Awareness is growing that fresh or minimally processed vegetables can be sources of pathogenic bacteria (Steele & Odumeru, 2004). Ready-to-eat salad items in supermarkets are becoming more readily available, salad bars in restaurants are proliferating and salad vegetables are being used more often as part of sandwiches and salads produced by the food service industry (Beuchat, 1996). As more consumers revert to healthier lifestyles, reducing their consumption of animal products and consuming minimally processed foodstuffs such as grains and legumes, vegetables and fruits, this change in dietary habits contribute to increasing outbreaks of food infections associated with consumption of raw produce (Alzamora *et al*., 2000). Foods such as these are often prepared by hand. Direct contact of food with handlers may lead to an increased incidence of contamination (Christison *et al*., 2008; Christiansen & King, 1971). Furthermore, the increase has been contributed to by factors such as adaptation of foodborne pathogens to environmental conditions, increase in international trade, and decreased use of chemical preservatives as healthier lifestyles are pursued along with the demand for higher convenience food products (FAO/WHO, 2006).

This recent recognition of fresh fruits and vegetables as major vehicles of foodborne illness has led to increased research on the occurrence of pathogens on fresh produce, the mechanisms by which they survive and persist in this adverse environment and the factors that enable them to grow and proliferate in the field and post-harvest. Several articles have been published that investigate these subjects, along with key factors that play a role in contamination and outbreaks related to fresh or minimally processed fruits and vegetables (Beuchat, 2002a; Brandl, 2006; Heaton & Jones, 2008; Lynch, *et al*., 2009; Teplitski *et al*., 2009; Tyler & Triplett, 2008). Fig. 1 summarises the epiphytic (on the outer surface) and endophytic (proliferating on interior of structure) sources of enteric foodborne pathogens (Critzer & Doyle, 2010).

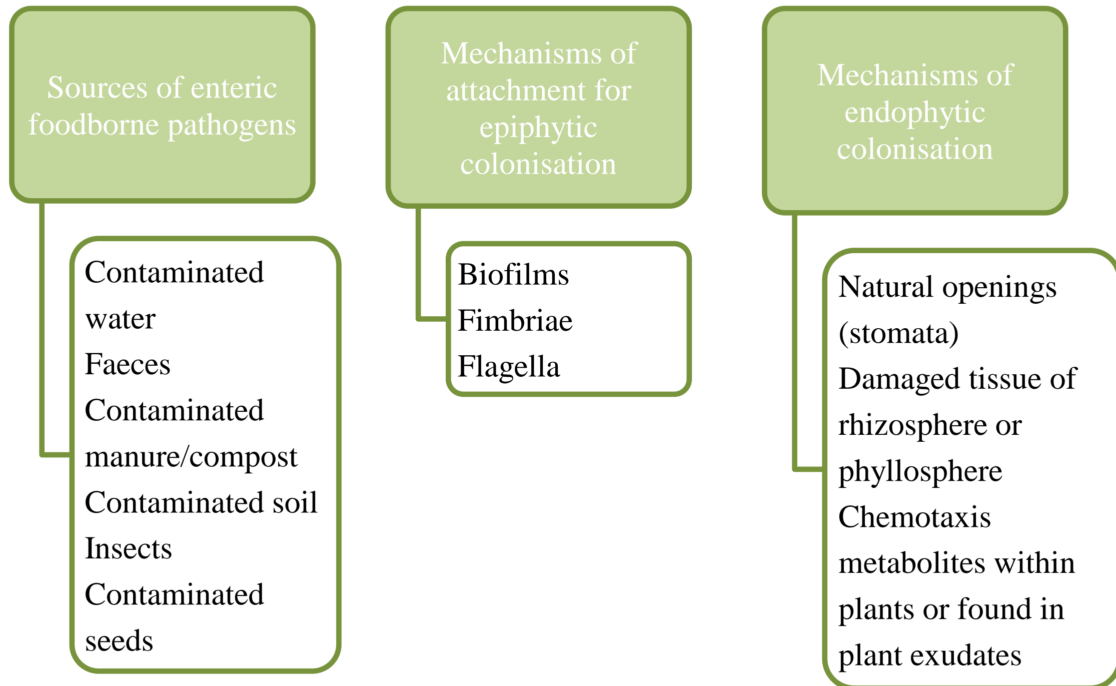



**Figure 1** Colonisation of fresh fruits and vegetables by enteric food borne pathogens (adapted from Critzer & Doyle 2010)

The risk of disease is increased when fruit and vegetables are consumed raw (Steele & Odumeru, 2004). Among the various irrigated crops, vegetables are some of the produce most vulnerable to contamination. As they are often eaten raw (uncooked) they can pose a significant threat to humans (Armon *et al.*, 2002). Ensuring microbiological safety and stability of such fresh produce is thus of utmost importance.

To induce foodborne illnesses by inferior irrigation water quality, a pathogen has to survive in the water, be transmitted to the edible part of the plant in a number corresponding to the infectious dose of the pathogen, survive on the edible plant part during the interval between irrigation and harvest and withstand postharvest handling (Heaton & Jones, 2008).

# 2.2. *LISTERIA MONOCYTOGENES*

## 2.2.1. Characteristics

*L. monocytogenes* is a gram-positive, non-spore forming, non-acid fast, rod-shaped bacterium that was first described in 1926 (Pearson & Marth, 1990). It is aerobic and facultatively anaerobic, catalase positive and oxidase negative (Peiris, 2005). The rods are 0.4-0.5 µm in diameter and 0.5-2 µm in length. Their motility is exhibited through flagella and the rods occur singly or in short chains. The genus *Listeria* includes six species: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seegligeri* and *L. grayi*. Of these, only *L. monocytogenes* is known to be causative of human illness. The organism is widely distributed throughout the environment and has the ability to grow in varying conditions, including at temperatures from 0.5 to 45°C, at a pH range of between pH 4.7 and 9.2 and over a wide range of osmotic pressures. It is therefore able to survive for long periods under adverse conditions (Peiris, 2005) and thrive in various niches including soil, sewage, plants and animals, food processing plants and home refrigerators. *L. monocytogenes* grows optimally at temperatures between 30 and 37°C (Pearson & Marth, 1990) and has been known to survive in plant materials for 10 to 12 years (Beuchat, 1996). The non-fastidious nature of the pathogen increases the risk of illness as a result of contamination of foods (Gorski *et al*., 2003).

When cultured on nutrient agar, the colonies are round and 0.5-1.5 mm in diameter, are translucent and have a smooth, glistening surface (Peiris, 2005). On Oxford *Listeria* Selective Agar, *L. monocytogenes* hydrolyses esculin to esculetin. This results in the formation of a black complex with iron(III) ions, producing brown-green coloured colonies with a black halo (Anon., 2009a). Brilliance™ *Listeria* Agar (formerly Oxoid Chromogenic *Listeria* Agar (OCLA)) is a medium also used for isolation and enumeration of *Listeria* species from food samples. This media uses the chromogen X-glucoside for presumptive identification of *Listeria* spp. This chromogen is cleaved by ß-glucosidase which is common to all *Listeria* species. *Listeria monocytogenes* is then further differentiated by its ability to produce the phospholipase enzyme, lecithinase, which hydrolyses the lecithin in the medium to produce an opaque white halo around the colony (Anon., 2009b).

*L. monocytogenes* is a pathogen that causes listeriosis when ingested by humans or animals and the consumption of contaminated foods has been considered as the primary source of infection. Although rare when compared to other foodborne diseases, listeriosis often leads to severe consequences (Buchrieser, *et al*., 2003), and despite its low incidence, the mortalities associated with outbreaks are high. This renders *L. monocytogenes* one of the most significant pathogens associated with foods (Warriner & Namvar, 2009). Although unknown, it is thought that the infective dose is approximately 100 - 1000 cells (Warriner & Namvar, 2009; Drevets & Bronze, 2008), which varies depending on the susceptibility of the host immune system, the organism's virulence, the type of food consumed and quantity thereof, as well as the concentration of the pathogen in the food. Consumption of food contaminated with levels as low as $10^2$ to $10^4$ cells per gram of food have been reported to have caused disease (Peiris, 2005). Clinical features of listeriosis include meningitis, meningo-encephalitis, septicaemia, abortion, perinatal infections and also gastroenteritis (Buchrieser, *et al*., 2003). The incubation period for illness varies from days to weeks and with infection in some listeriosis outbreaks having been reported to develop one day after the contaminated food was eaten (Peiris, 2005).

*L. monocytogenes* was only considered a significant foodborne pathogen from 1981 when, during an outbreak, the bacterium was linked to contaminated coleslaw (Warriner & Namvar, 2009). According to the United States Centre for Disease Control, as on the 7th August, 411 cases of listeriosis had been reported in the year 2010, with an average of 21 cases having been reported per week over the 5 preceding years. It was reported that 1097 disease outbreaks occurred in the United States during 2007 (the most recent finalised data as on August 13th, 2010), which resulted in 21,244 cases of foodborne illness and 18 deaths. Among the 18 reported deaths, three were attributed to *L. monocytogenes.* Leafy vegetables were said to have caused 14% of the illnesses attributed to a single food commodity (CDC, 2010).

### 2.2.2. *L. monocytogenes* and vegetables

The microbiology of fresh produce falls in an area that lacks in knowledge and this deficiency of information influences the safety of fresh produce markedly (Nguyen-the and Carlin, 1994; Jacxsens *et al*., 2009; Alsanius *et al*., 2010) as greater

understanding of the specific behaviour of pathogens, their locations in and on plant surfaces can aid the development of means to treat products to eliminate these pathogens (Brackett, 1999a).

The involvement of foods such as smoked meats and dairy products as vector for transmission of *L. monocytogenes* has on numerous occasions been established (Buchrieser *et al*., 2003, Schuchat *et al*., 1991) and fresh produce has also been implicated in cases of *L. monocytogenes* contamination.

A field study of the microbial quality of fresh produce by Johnston *et al*., (2005) demonstrates that the microbial load of produce may be affected by every step from production to consumption. Vegetables can become contaminated with pathogenic microorganisms by contact with soil, through irrigation or during postharvest washing with contaminated water or by contact with infected food handlers (Beuchat & Ryu, 1997). There are several pathways with which *L. monocytogenes* can be transmitted from plants to humans via vegetables. These include carry-over from animals and their faeces, to sewage which runs into water that is taken up by soil, which in turn comes into contact with vegetables or the pathogen may be transferred post-harvest during handling in processing environments to the vegetables that are finally consumed (Beuchat, 1996).

Although pathogens such as *L. monocytogenes* are commonly found in the environment, their presence is indicative of recent human or animal faecal contamination (Steele & Odumeru, 2004). Surveys have been performed in several countries to determine local prevalence of pathogenic microorganisms including *L. monocytogenes*, on fruit and vegetables (Arumugaswamy *et al*., 1994; De Simon *et al*., 1992; Heisick *et al*., 1989a; Little *et al*., 1999; McMahon & Wilson, 2001; Odumeru *et al*., 1997; Pingulkar *et al*., 2001; Sagoo *et al*., 2001; Szabo *et al*., 2000; Tang, *et al*., 1994; Thunberg *et al*., 2002; Wong *et al*., 1990).

In a case study in the United States, *L. monocytogenes* was found in 6 of 127 vegetables samples (4.7%) from farmers and supermarkets (Thunberg *et al*., 2002) and a survey of 1000 samples of 10 types of fresh produce in the same country isolated *L. monocytogenes* from cabbage, cucumbers, potatoes and radishes (11.4% of samples tested) (Heisick *et al.,* 1989b). Three of 890 fresh produce samples in Norway were found to contain *L. monocytogenes* (Johannessen *et al*., 2002). In similar fresh produce surveys, prepared mixed salads displayed a high rate of contamination and this was attributed to cross contamination of vegetables with the

pathogen during handling, including chopping, mixing and packaging. *L. monocytogenes* was detected in almost half of fresh cut vegetable samples in a survey carried out in The Netherlands and 7 out of 66 prepared vegetable salads in a survey in Northern Ireland were reported to contain the organism (Beuchat, 1996). *L. monocytogenes* was detected in 10.6% of samples of washed produce in India (Pingulkar *et al.*, 2001), in 22% (5 of 22) of leafy vegetables in Malaysia (Arumugaswamy *et al.*, 1994) and in 8 of 103 (7.8%) vegetable samples in Spain (De Simon *et al.*, 1992), as well as in 20% of 50 cabbage salad samples in Costa Rica (Steele & Odumeru, 2004). The presence of *L. monocytogenes* in food processing environments is acknowledged and the production of minimally processed foods free of the pathogen is complicated by the ubiquity of the pathogen in nature (Taormina & Beuchat, 2001).

A large outbreak of listeriosis that occurred across Canada in 2008 was referred to in a review by Warriner and Namvar (2009), as a phase of "*Listeria* hysteria", involving 41 cases, including 18 deaths. Beuchat (1996), reported on an outbreak of listeriosis in 1979, which involved 23 patients from eight different hospitals. In this particular incidence, it was concluded that the consumption of raw vegetables could have been the cause of the illnesses. Another case of listeriosis reported that *L. monocytogenes* was isolated from commercially prepared, unopened coleslaw after prolonged refrigeration and was also found on various other forms of fresh produce, including celery, carrots and cucumber (Beuchat, 1996).

Numerous other disease outbreaks linked to contaminated vegetables have been summarised in reviews (Beuchat, 1996; Beuchat & Ryu, 1997; Long *et al.*, 2002; Steele & Odumeru, 2004) and specific produce, such as cabbage, lettuce and celery, have even been associated with the presence of *L. monocytogenes* in cases of listeriosis (Aureli *et al.*, 2000; Ho *et al.*, 1986; Schlech *et al.*, 1983). The organism has furthermore been responsible for the recall of red peppers, sprouts, and potato salad (Gorski *et al.*, 2003; Brackett, 1999a) and surveys from U.S. grocery stores have revealed the presence of *L. monocytogenes* on radishes, potatoes and cucumbers (Heisick *et al.*, 1989a). Others have not only isolated it from asparagus, lettuce, parsley, watercress as well as cauliflower, broccoli and other leafy vegetables (Porto & Eiroa, 2001; Weis & Seeliger, 1975), but have suggested that *L. monocytogenes* can grow or survive on such fresh or processed produce (Berrang *et al.*, 1989; Carlin & Nguyen-the, 1994; Farber *et al.*, 1998; Heisick *et al.*, 1989b; Lin *et al.*, 1996).

Christison *et al*. (2008), performed a microbiological surveillance and monitoring survey of ready-to-eat foods in retail delicatessens in Johannesburg, South Africa and found that 4% of salad samples tested positive for *Listeria monocytogenes*, a result which was comparable to the estimation of 5% incidence of the pathogen in delicatessen style salads in other studies (Guerra *et al*., 2001, Goulet *et al*., 2001; Uyttendale, *et al*., 1999). The only produce items that seem to inhibit growth of *L. monocytogenes* are tomatoes and carrots (Beuchat & Brackett, 1991; Nguyen-the & Lund, 1991).

Despite vegetable consumption on several occasions being linked to listeriosis outbreaks, the fact that the incidence rates reported are generally below 10%, has rendered vegetables to be considered of low risk, resulting in few studies focusing on the investigation of the presence of *L. monocytogenes* in this food type, regardless of the ubiquity of this microorganism, and the fact that vegetables seem to be a good substrate for growth of *L. monocytogenes* (Aguado *et al*., 2004).

One such vegetable substrate is broccoli. Broccoli (*Brassica oleracea* var *botrytis)* is a type of cabbage crop, grown in the winter with a water requirement of between 30 and 38 mm of water per week. Most broccoli harvested in the United States is sold as fresh produce and the increased awareness of the beneficial effect of minimally processed vegetable produce on health, amongst other factors, has caused a rapid increase in the demand for fresh broccoli (Cliff *et al*., 1997). The surface of a broccoli floret is rough and the crevices in the broccoli structure (Frank, 2001), retain water and aid in attachment of organisms to the crop, therefore making this vegetable a possible substrate for organisms such as *L. monocytogenes* (Stine *et al*., 2005). Broccoli is a crop that grows close to the ground, making it more likely to contain *Listeria* as the crop may come into contact with soil, facilitating transfer of the pathogen onto the crop surface (Gorski *et al*., 2003).

The regulatory authorities of countries such as France and Germany stipulate a limit for *L. monocytogenes* of 2 log cfu/g of vegetables (Nguyen-the & Carlin, 1994). The United States has classified *L. monocytogenes* an adulterant and the United Kingdom requires absence of *L. monocytogenes* on 25 g of ready-to-eat food to render it suitable for human consumption (Francis *et al*., 1999). A zero-tolerance approach to *Listeria* control is also applicable in Austria, Australia, New Zealand as well as Italy (Warriner & Namvar, 2009). Guidelines published by the South African Department of Health state that *L. monocytogenes* should be absent in one gram of

raw vegetable produce (Lötter, 2010), meaning that the detection thereof on a food would trigger a product recall.

### 2.2.3. Attachment, growth and survival of *L. monocytogenes*

The fundamental question of which factors are responsible for the initial association of *L. monocytogenes* with produce and how the organism is able to remain attached have not been addressed by many studies (Gorski *et al*., 2003) and enhanced knowledge on the ecology of the pathogen in fresh produce would enable the development of more effective disinfection strategies (Brackett, 1999b).

#### 2.2.3.1. Attachment

Microorganisms attach to surfaces by means of fimbriae (pili), fibrils and flagella interacting with surfaces by means of electrostatic, hydrogen bonding and hydrophobic forces, followed by production of exocellular binding polymers (Frank, 2001). Attachment to broccoli is possible due to the large contact surface of the vegetable and the crevices in the broccoli, which retain water, providing more contact time and so aiding in attachment to the rough surface of the broccoli (Stine *et al*., 2005). Attachment takes place at the stomata, broken trichomes or cracks in the cuticle. The surface layers of a plant are illustrated in Fig. 2.

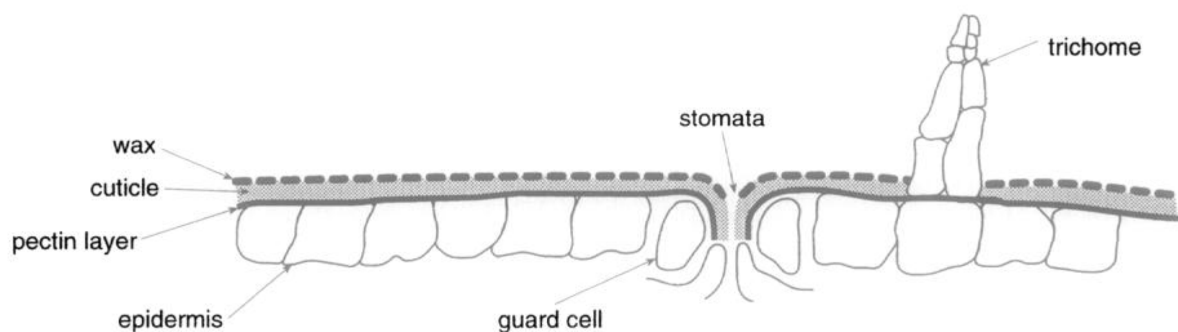



**Figure 2** Surface layers of a plant (Frank, 2001)

In the process of colonisation of plant surfaces, attachment of enteric pathogens is the first step, allowing the organism to settle in the non-host environment (Critzer &

Doyle 2010). Even though flagellar filaments have been demonstrated to be involved in surface binding of *L. monocytogenes* and that strains demonstrating enhanced attachment produced extracellular fibrils (Kalmokoff *et al*., 2001; Vatanyoopaisarn *et al*., 2000), Solomon and Matthews (2006) showed that gene expression, motility or production of extracellular compounds, were not necessary for initial attachment. .In their study of microbial attachment under model conditions, Kalmokoff *et al*. (2001), found that such bacterial processes are, however, not only likely to be important for extended survival of the pathogen on the leaf (Heaton & Jones, 2008) but also that attached cells are much more resistant to cleaning. Their findings indicated that there was very little difference among *L. monocytogenes* isolates in terms of the ability of cells to adsorb to a plant surface. Gorski *et al*. (2003), studied the molecular interactions with plant tissue influencing the attachment of *L. monocytogenes* on radishes and their results indicated that temperature may play a key role in the bacterial processes that govern the type and function of attachment factors available to the pathogen.

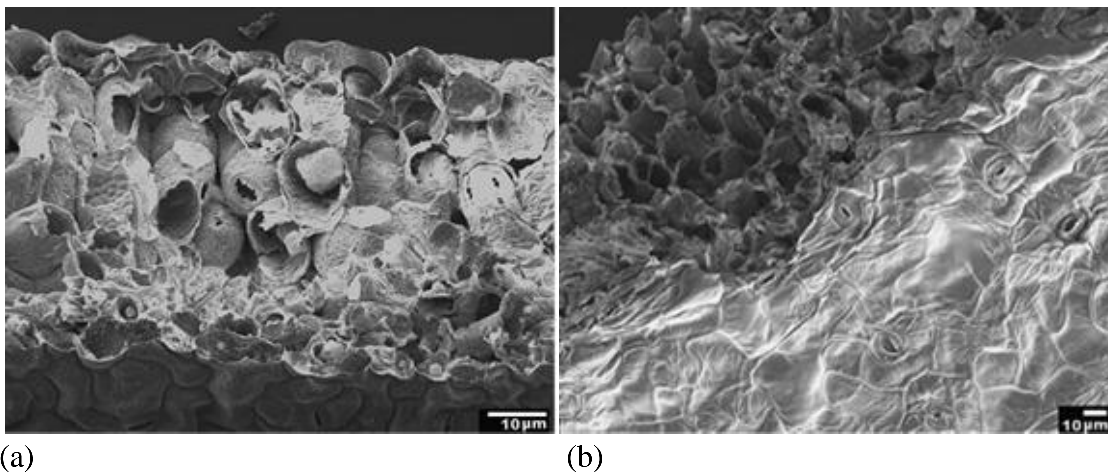
In response to various lines of evidence indicating that *L. monocytogenes* contamination of fruits and vegetables may contribute to the burden of human listeriosis infections, Milillo *et al.* (2008), assessed the ability of *Listeria monocytogenes* to attach to and grow on *Arabidopsis thaliana*, a well characterized plant model. The *Arabidopsis thaliana* plant is a small flowering plant popularly used as a model organism in biological studies as representation of plant interactions with microorganisms. Using this plant model system, of which the small genome sequence has been completed, the researchers gained a better understanding of pre-harvest interactions between *L. monocytogenes* and plants. Their data indicated that *L. monocytogenes* is able to rapidly attach to and proliferate on *A. Thaliana* after inoculation of leaves which was found to be due to the ability of *L. monocytogenes* to survive and multiply under the typical stress conditions encountered on plant surfaces. (Milillo *et al*., 2008).

2.2.3.1.1. *Scanning Electron Microscopy*

Scanning Electron Microscopy (SEM) is often used to examine surfaces (Kalab *et al*., 1995), and is a technique that is ideal for high resolution examination of plant surfaces (Baker & Holloway, 1971). The sample is dried as the absence of water exposes their

solid structures for examination. A 5-20 nm thick gold coating facilitates electric conductivity, scanning the sample by focusing an electron beam. The electrons are processed to form an enlarged image which is easily understandable and has a great depth of focus (Kalab *et al*., 1995). Kalmokoff *et al*. (2001), described the sample preparation for study with SEM.

Scanning Electron Microscopy has been used for countless studies, to investigate plant surface structure as well as microbial attachment to surfaces and has therefore received great attention and been of great value in studies of food products and microbial colonisation. Ren *et al*. (2007), investigated the surfaces of several typical plant leaves by scanning electron microscopy to describe different non-smooth surface characteristics, while Baker & Holloway (1971), studied the waxes on plant surfaces. SEM has been proven to be a technique of great versatility with which the micro-topography of waxy plant surfaces can be rapidly studied. Images of SEM studies on broccoli are illustrated in Fig. 3.




(a) (b)

**Figure 3** Broccoli leaf surface sample fracture preserved using the (a) Critical Point Drying technique, (b) Freeze drying technique (liquid nitrogen) (Pathan *et al*., 2008).

Individual cells present on various surfaces have been demonstrated on scanning electron micrographs (Kalmokoff *et al*., 2001; Herald and Zottola, 1988; Blackman and Frank, 1996). According to Critzer & Doyle (2010), foodborne pathogens form biofilms on plant tissue during colonisation of the surface and biofilms have been found present on cotyledons, hypocotyls, and roots of commercially purchased broccoli examined for microbial attachment by scanning electron microscopy (Fett, 2000). In addition to enabling the organisms to survive in the harsh conditions, the

presence of pathogens in biofilms on crops may decrease the effectiveness of sanitisers such as chlorine.

Arnold & Bailey (2000), studied bacterial attachment to stainless steel surfaces in food production areas and on processing equipment by means of SEM, while the ability of isolates of *L. monocytogenes* to adsorb and adhere (and form biofilms) on a food-grade stainless steel surface was investigated by Kalmokoff *et al*. (2001), and Borucki *et al*. (2003), as illustrated in Fig. 4.

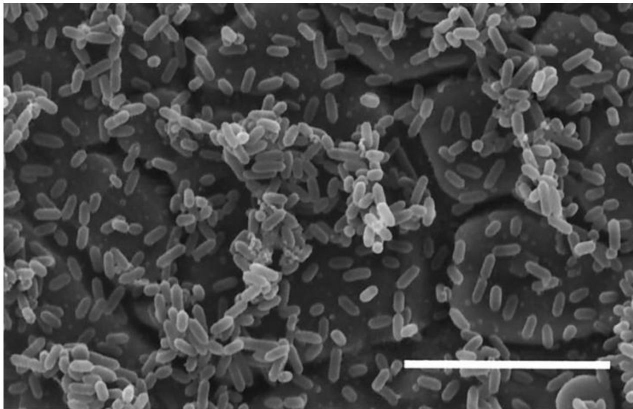


**Figure 4** *L. monocytogenes* on stainless steel (Borucki *et al*., 2003)

SEM was further used to examine the location at which microorganisms invade spinach leaves (Babic *et al.,* 1996), and bacterial behaviour on carrot and lettuce (Gleeson & Beirne, 2005). Gorski *et al*. (2003), investigated the attachment of *L. monocytogenes* on radishes.

### 2.2.3.2. Survival

If a pathogen manages to persist on the surface of a crop, a chance exists that the organism may remain on the plant at an infective dose level at point of consumption (Heaton & Jones, 2008).

The survival of *L. innocua* on parsley leaf surfaces via direct inoculation was measured by Girardin *et al*. (2005). Their results indicated that *L. innocua* populations directly inoculated on parsley leaves decreased from 1.4 x $10^7$cfu/g to less than 0.23 cfu/g within 48 h under field conditions. A rapid decrease of 6 log cycles occurred within the first 5 hours, followed by a slower decline of the remaining fraction of the population (2 log cycles within 43 h). In spite of the high inoculum

applied to the leaves, no *Listeria* could be detected after 48 h. The authors suspected that the use of laboratory maintained strains of *L. innocua* may explain the poor persistence of the organism observed under environmental conditions (Girardin *et al*., 2005).

Once internalised, the possibility arises that enteric pathogens may escape the effect of surface disinfectants for fresh produce, as was noted by Critzer & Doyle (2010), in reviewing the microbial ecology of foodborne pathogens associated with produce. *L. monocytogenes* was found not to be internalised in seedlings evaluated after inoculation onto plant seeds, but the organism did persist on the plant surface throughout the cultivation period (Jablasone *et al*., 2005). According to Beuchat (2002b), the success of infiltration of pathogens into fruit and vegetable tissues is dependent on various conditional factors, including the environmental temperature and the time that the organism remains in contact with the plant surface, only occurring once the water pressure on the produce surface overcomes internal gas pressure and the hydrophobicity of the produce surface.

#### 2.2.3.3. Environmental parameters

The growth and death of microorganisms responsible for foodborne disease are significantly influenced by environmental factors, including temperature (Duh & Schaffner, 1993). Girardin *et al*. (2005), quantitatively assessed the survival of *L. innocua* under field conditions and found that it survived better in winter, indicating an important influence of environmental temperature. According to Steele & Odumeru (2004), cooler temperatures promote survival of pathogenic microorganisms on fruit and vegetables and the majority of *L. monocytogenes* isolates found on cabbage by Prazak *et al*. (2002a), were found during the winter growing season.

Pathogens present on vegetables during crop growth are killed if they are exposed to unfavourable climatic conditions and this inactivation is more rapid in hot, sunny weather than in cool, cloudy or rainy conditions, with wet conditions favouring pathogen survival (Keraita *et al*., 2007). Most of the above mentioned authors suggested that more research into the effects of seasonal conditions on pathogens present on produce and variations due to seasonality is required.

## 2.2.4. Effect of processing on *L. monocytogenes*

The presence of *L. monocytogenes* in processing plants is becoming an issue of increasing importance. During processing the pathogen can easily contaminate food, with the food preparation environment representing a significant niche for *L. monocytogenes*. The concern is fuelled by the fact that, once established, eliminating *Listeria* from the food processing environment becomes an impossibility rather than a mere difficulty (Warriner & Namvar, 2009). Food serves as a reservoir and vehicle for *L. monocytogenes* to enter the digestive tract of consumers (Chae & Schraft 2000).

### 2.2.4.1. Minimal processing

With focus on a healthy lifestyle gaining importance amongst consumers, the demand for foods retaining their natural sensory properties such as flavour, colour and texture and containing fewer preservatives, has risen remarkably (Ramesh *et al*., 2002; Alegre *et al*., 2010). Minimal processing technologies are designed to limit the impact of processing on nutritional and sensory quality and to achieve extended shelf life without the use of synthetic additives (Ohlsson & Bengtsson, 2002). The disadvantage, however, is the increased risk of the survival of certain harmful organisms, in particular *L. monocytogenes*, as this organism can survive in extreme environments, including at refrigeration temperatures (Peiris, 2005) and under the oxygen concentrations within modified atmosphere packaging (MAP) (Francis *et al*., 1999).

Minimally processed vegetables are classified as those that have been either trimmed, peeled, sliced or shredded and washed or treated with sanitizer. Such ready-to-eat (RTE) products are packaged and often stored at refrigeration temperatures. Organisms present on such vegetables may remain on the produce even after minimal processing and the possible presence of which pathogens within this microflora poses a safety risk to consumers (Francis *et al*., 1999).

2.2.4.1.1. *Washing*

During minimal processing, RTE vegetables are washed and usually dipped in water containing a washing agent. Washing with water has been reported to remove unattached organisms (Ells & Hansen, 2006, Gorski *et al*., 2003), but including antimicrobials such as chlorine in the wash water improves the efficacy of the washing process (Francis *et al*., 1999). As *L. monocytogenes* attaches to broccoli in natural plant contours, openings and crevices of the structure (Frank, 2001), it may however resist being washed off by water and be protected from the effect of chlorination (Francis *et al*., 1999), escaping the antimicrobial effect of postharvest washing (Steele & Odumeru, 2004).

*L. monocytogenes* is reported to have remained viable on brussel sprouts dipped for 10s in water containing 200 μg/ml of chlorine and from this Beuchat (1996), concluded that the removal of *L. monocytogenes* from contaminated vegetables by means of hypochlorite was ineffective. A study on the role of leaf structure in protecting pathogens present on lettuce indicated that cells contained within the stomata of lettuce and within the trichome and in cracks of the cuticle survived chlorine treatment (200 ppm), as these cells were protected from the chlorination effect (Critzer & Doyle 2010; Takeuchi & Frank, 2001). Although chlorine treatment may be beneficial for reducing food borne pathogen contamination, Johnston *et al*. (2009), in their study of lettuce and spinach, suggested that it may also decrease the population of organisms acting as beneficial antagonists.

Treatment of *L. monocytogenes* cells with chlorine at concentrations of 2.0, 2.4 and 6.0 mg/l resulted in injury of cells and treatments of 10 min reduced cell numbers by 0.62, 1.30, or 4.02 log units, respectively. Zhang & Farber (1996), also reported on the effects of disinfectants against *L. monocytogenes* on fresh-cut vegetables and observed a reduction after chlorination on lettuce and cabbage. After studying the effectiveness of washing methods in removing *L. monocytogenes* from fresh produce, Prazak *et al*. (2002b), suggested that postharvest washing of cabbage could be beneficial in that it may reduce or eliminate contamination of products, but that chlorine does not eliminate pathogenic bacteria. Taormina and Beuchat (2001), suggested a possible sensitisation of cells to heat by chlorine, after establishing that treating cells with chlorine for 10 min caused more rapid death during subsequent heating than did treating cells with chlorine for 5 min.

As a strong oxidant, chlorine presents antimicrobial action by inhibiting bacteria by means of an irreversible oxidation of sulphydryl groups, interfering in cell metabolism. It induces leakage of macromolecules from the cells indicating permeability changes of the membrane (Venkobachar *et al*., 1977).

2.2.4.1.2. *Modified Atmosphere Packaging*

Fresh produce is packaged under conditions of modified atmosphere to delay ripening and reduce respiration and ethylene production, taking into account the natural process of respiration as well as the gas permeability of the package. These processes lead to an increase of carbon dioxide and a reduced oxygen concentration (Serrano *et al*., 2006) in order to extend the shelf life and quality thereof by slowing down product respiration and microbial growth as well as delaying physiological aging (Francis *et al*., 1999).

It has been found that optimal MAP conditions for broccoli are a concentration of 5% $CO_2$ and below 2% $O_2$ (Zagory & Kadel, 1988). According to Ishikawa *et al.* (1998), these MAP conditions can be obtained by packaging in an LDPE (Low Density Polyethylene) film with properties as indicated in Table 1, or by gas flushing, with optimal gas concentrations as indicated in Table 2, usually sealed within semi-permeable packages.

**Table 1** Film properties for optimal package conditions of broccoli (Ishikawa *et al*., 1998)

| **Film** | **Thickness (µm)** | **Gas transmission rate (cc/day/atm)** | | | **Surface area ($m^2$)** |
|---|---|---|---|---|---|
| | | **$N_2$** | **$O_2$** | **$CO_2$** | |
| LDPE | 29 µm | 1100 | 5700 | 27 500 | 0.1998 |

**Table 2** Oxygen, carbon dioxide and nitrogen concentrations for controlled atmosphere storage (Ishikawa *et al*., 1998)

| **Gas concentration (%)** | | |
|---|---|---|
| **$O_2$** | **$CO_2$** | **$N_2$** |
| 2 | 5 | 93 |

Serrano *et al*. (2006), found that storing broccoli heads under a modified atmosphere resulted in a five-fold increase in storability of broccoli in terms of quality, with Cliff *et al*. (1997), observing a decrease in $O_2$ and an increase in $CO_2$ levels during storage.

Despite the reported benefits of MAP storage on broccoli quality, storage in PVC or cling film is still commonly used as broccoli packaging material (Jacobsson *et al*., 2004). The wider use of the MAP method continues to be limited by the concern that pathogenic bacteria may potentially survive and grow at refrigeration temperatures (Nguyen-the & Carlin, 1994). Some studies have shown that *L. monocytogenes* is not greatly inhibited by vacuum or by $CO_2$-enriched atmospheres and can therefore remain able to proliferate at the reduced temperatures encountered during refrigeration (Kakiomenou *et al*., 1998). While the inhibition of listerial multiplication during packaging under modified atmospheres has on occasion been reported by other authors, in general, these conditions seem not to have a bactericidal effect on the pathogen (Garcia de Fernando *et al*., 1995). After an investigation on the behaviour of *L. monocytogenes* on raw broccoli under modified atmosphere gas packaging by Beuchat (1996), MAP appeared to have little or no effect on the rate of growth of the organism on the vegetable. Kakiomenou *et al*. (1998), determined the effect of a modified atmosphere on the predominance of *L. monocytogenes* on vegetables and found that the organism survived but did not grow in packages with initial head-spaces of 4.9% $CO_2$, 2.1% $O_2$, 93% $N_2$ and 5% $CO_2$, 5.2% $O_2$, 89.8% $N_2$. They further concluded that modification of the atmosphere could not be considered as the only factor involved in the inhibition of *Listeria* because changes in type of vegetable, initial pH and competition with other flora also affect their growth.

2.2.4.1.3. *Cold storage*

Food that is held in prolonged cold storage before distribution may allow proliferation of the psychrotrophic *Listeria monocytogenes* while the number of competing microorganisms decreases (Pearson & Marth, 1990). The ability to grow at low temperatures is central to the persistence of *L. monocytogenes* in food processing environments. Warriner & Namvar (2009), interestingly made the estimation that the prevalence of *Listeria* in domestic fridges is 20%.

During a study of the behaviour of *L. innocua* during production of parsley, the organism was found to survive at low temperature (Girardin *et al*., 2005). Wonderling *et al*. (2004), reported that *L. monocytogenes* will grow at refrigeration temperatures and Heaton & Jones (2008), even commented that *L. monocytogenes* is likely to multiply during storage if present on fresh produce. Schoeller *et al*. (2002), found that *L. monocytogenes* inoculated on sprouts increased by 0.75 log cfu/g during 9 days of refrigerated storage. They also noted that a lower initial pathogen level resulted in no significant change over the storage period. Flessa *et al*. (2005), observed a reduction of approximately 3 log cycles on strawberries after 7 days refrigerated storage.

Duh & Schaffner (1993), did however conclude that refrigerated storage alone cannot ensure that the growth of *L. monocytogenes* will not occur.

### 2.2.4.2. Microbial interactions

Microbial interactions with foodborne pathogens on fresh produce has been reviewed by Critzer & Doyle, (2010), who observed that the inoculation of *Lactobacillus casei* onto Scarola lettuce was coupled with a reduction in the population of *L. monocytogenes* by at least 2 log cfu/g upon simultaneous inoculation and subsequent storage at 8°C for six days. The natural microflora of minimally processed produce, including carrots, green peppers, lettuce, cabbage, celery and onions were found to be inhibitory to *L. monocytogenes* by Schuenzel & Harrison, (2002). Heaton & Jones (2008) have also suggested that, when competing for the same carbon source, *L. monocytogenes* present on vegetables in the field may be outcompeted by background flora, preventing the pathogen's growth (Carlin *et al*., 1996).

The mechanisms that can be implemented to control food borne pathogens with the use of natural microflora can assist in developing effective inhibition microflora that alleviate the colonisation and mitigate pathogen survival on fresh produce (Scolari & Vescovo, 2004).

### 2.2.4.3. Heat treatment

#### 2.2.4.3.1. *Cooking*

During blanching, vegetables are subjected to a heat treatment of 100°C in boiling water for a brief interval, this being the recognised method of effectively combating enzymes in food products (Ramesh *et al*., 2002). As early as 1971, it was recommended that broccoli should be blanched in boiling water for three to four minutes or in steam for six to eight minutes from a sensory point of view. Microwave blanching causes less loss of nutrients due to leaching than conventional steam or water blanching of vegetables, therefore resulting in better retention of nutrients (Ramesh *et al*., 2002).

Mazzotta (2001), studied the heat resistance of *L. monocytogenes* by submitting broccoli florets to a blanching treatment and found that blanching can be used as an antilisterial treatment if the cold spot of vegetables is treated for at least 10 s at 75°C or instantaneously (< 1s) at temperatures above 82°C.

Farber *et al*. (1998), observed survival of *L. monocytogenes* at temperatures up to 67.5°C, but not above, in milk and Coote *et al*. (1991), determined that maintaining a temperature of 70°C for at least 2 min throughout a food substantially reduces *L. monocytogenes numbers*. Lund *et al*. (1989), also indicated that heating to this temperature caused a $10^6$-fold lethality to *L. monocytogenes*.

#### 2.2.4.3.2. *Microwave heating*

Microwave ovens have become not only common but essential appliances in many kitchens. This heating technique offers benefits of faster cooking times and energy savings over conventional cooking, resulting in widespread use of microwaves for cooking (Venkatesh & Raghavan, 2004). Broccoli is a fragile food in terms of sensory and texture characteristics and the use of microwave cooking has been recommended to better maintain sensory quality (Smith & Williams, 1971).

Understanding the mechanism of microwave heating assists in predicting and explaining the heating effect that this cooking technique has on food products as well as on microorganisms. Microwaves belong to the portion of the electromagnetic

spectrum with wavelengths from 1 mm to 1 m with corresponding frequencies between 300 MHz and 300 GHz. Two frequencies are commonly used for microwave heating: 0.915 and 2.45 GHz. In conventional thermal processing, energy is transferred to the material due to thermal gradients, through convection, conduction, and radiation of heat from the surfaces of the material. The energy inside a microwave oven cavity is an oscillating electrical and magnetic field and microwave energy is delivered directly to materials through molecular interaction with the electromagnetic field and converted to thermal energy within the product, as explained by Ramesh *et al*. (2002), through energy conversion, rather than heat transfer (Thostenson & Chou, 1999).

Ramesh *et al*. (2002), compared pulsed microwave blanching with conventional water blanching at 95±2°C. During microwave blanching, heat is internally developed at the centre of the food, whilst heat should penetrate the vegetable tissue in the case of water blanching. This explains their findings that during microwave blanching, the time it took to reach an internal vegetable temperature above 90°C was markedly less (40 s) than the 125 s it took during water blanching.

Several studies on microwave power to numerous food processes, including blanching (Avisse & Varoguaux, 1977), both independently and combined with steam or water heating (Collins and McCarty 1969; Huxsoll *et al*., 1970; Ramaswamy & Fakhouri 1998) and cooking (Decareau, 1985; Suzuki & Oshima, 1973), have been reported (Ramesh *et al*., 2002). Microwave heating is known to assist in the extension of food preservation by inactivating and so facilitating reduction of many microorganisms, including *Listeria* spp. (Venkatesh & Raghavan, 2004).

Woo *et al*. (2000), observed that with an increase in microwave temperature, viable cell counts in suspensions decreased substantially. The leakage of nucleic acid and protein from cells indicates damage to the cell structure. In their examination of the mechanism of microbial cell inactivation by microwave heating, Woo *et al*. (2000), observed that when the temperature reached 60°C, a 2-log reduction in Gram positive (G+) organisms occurred. The cell density was found not to decrease, which implies that G+ cells did not suffer membrane damage. The fact that no damage to the surface structures of the cells was observed, suggested that the microwave-radiated cells remained unlysed in suspension, despite being inactivated by the radiation (Woo *et al*., 2000). The results of Coote *et al*. (1991), showed that when

heating to reach a temperature of 70°C throughout a food for a minimum of 2 min in the microwave, the numbers of *L. monocytogenes* are dramatically diminished. Because foods such as vegetables are not very thick, non-uniform heating is not generally a problem. The reason that pathogens sometimes survive microwave heating temperatures could, however, still be ascribed to creation of cold spots upon uneven heating by microwave ovens (Ramesh *et al*., 2002).

### 2.2.5. Surrogate organism: *Listeria innocua*

*Listeria innocua* is an organism found widely and naturally in the environment, including in soil. It displays close relation to the foodborne pathogen *Listeria monocytogenes* (Buchrieser *et al*.,, 2003), but is non-pathogenic in character (Girardin *et al*., 2005). It is thus often used as surrogate organism to study *L. monocytogenes* (O'Bryan *et al.,* 2006), without the safety risk. *L. innocua* lacks the 10-kb virulence locus that engenders pathogenicity to *L. monocytogenes* and this explains why *L. innocua* does not infect humans or animals and is regarded as non-pathogenic (Hof & Hefner, 2005). Girardin *et al*. (2005), studied the behaviour of *L. monocytogenes* during production of parsley by using *L. innocua* as non-pathogenic surrogate in order to work under field conditions and not in laboratory microcosms, so the actual behaviour of the pathogen in the field would be reflected. As *L. innocua* and *L. monocytogenes* are naturally present in the environment and are frequently associated, suggesting that these two species have similar ecological requirements (MacGowan *et al*., 1994; Aguado *et al*., 2004; De Luca *et al*., 1998), the authors assumed that the behaviour of the surrogate *L. innocua* is similar to that of the pathogenic species *L. monocytogenes*. The potential of *L. innocua* as surrogate for *L. monocytogenes* was investigated by a studying the heat resistance of the organisms in meat and poultry after which Fairchild and Foegeding (1993), proposed the use of *Listeria innocua* as a non-pathogenic surrogate for *L. monocytogenes* for thermal resistance studies in milk (O'Bryan *et al*., 2006).

Kamat & Nair (1996), identified *L. innocua* as possible biological indicator for the inactivation of *L. monocytogenes* during processing procedures, as they showed similar physical responses to many processing treatments.

*L. innocua* has also been used as surrogate organism for *L. monocytogenes* in many other studies on food products, including apple juice (Corte *et al*., 2004), meat (Castellano *et al*., 2004), milk (Brinez *et al*., 2006, Bermudez-Aguirre *et al*., 2009), fruits and vegetables (Kozempel *et al*., 2002), such as fresh cut apples (Karaibrahimoglu *et al*., 2004), and fresh cut packaged leafy salads (Scifò *et al*., 2009), as well as for processing studies (Buzrul & Alpas, 2004), and as a model in inhibition and other studies (Nakai & Siebert, 2004; Houtsma *et al*., 1994; Ter Steeg *et al*., 1995). Duh & Schaffner (1993), found that, in their study of the growth rate of *L. monocytogenes* and *L. innocua*, data from the two organisms were similar.

## 2.3. INFLUENCE ON AGRICULTURE AND THE ECONOMY

South Africa is the leading exporter of vegetables in Sub-Saharan Africa (Department of Agriculture, Forestry and Fisheries, 2009), with the demand for fresh vegetable produce still continually increasing nationally and globally (Ndiame & Jaffee, 2005; Alsanius *et al*., 2010). Fresh produce therefore plays a very important role in the country's agricultural industry and economy. The evaluation of the microbiological safety of such produce is essential, as any outbreaks of foodborne illness resulting from consumption of fresh produce originating from South Africa could lead to loss of confidence in the country's products and subsequent banning of such products for export.

## 2.4. OBJECTIVES AND HYPOTHESES

### *2.4.1. Objectives*

**Objective 1**

To determine the survival of *Listeria innocua* as surrogate organism to *L. monocytogenes* on spray irrigated broccoli.

**Objective 2**

To determine the effect of minimal processing and cooking on *Listeria innocua* as surrogate organism to *Listeria monocytogenes* present on broccoli.

### *2.4.2. Hypotheses*

**1st Hypothesis**

*Listeria monocytogenes* will survive on broccoli due to its ability to withstand a wide range of environmental conditions including high moisture concentrations, low oxygen concentrations and low temperatures (Francis *et al*., 1999). It grows optimally at 30 to 37°C, but can survive from below 3 to up to 45°C (Pearson & Marth, 1990). It can sense the surrounding oxygen concentration by means of proteins (Patschkowski *et al*., 2000), regulate respiration by controlling gene expression and adjust cellular water content with the help of compatible solutes to balance its environmental osmolality (Bremer & Krämer, 2000).

**2nd Hypothesis**

*Listeria monocytogenes* present on broccoli will survive minimal processing procedures, as it is able to grow at temperatures as low as 0.5°C due to the ability of the organism to adjust its membrane lipid composition as a cold shock response (Phadtare *et al*., 2000). It is facultative anaerobic and therefore capable of growth under the oxygen concentrations within modified atmosphere packaging in minimal processing (Francis *et al*., 1999). As it attaches to the broccoli, also in crevices of the structure (Frank, 2001), it resists from being washed off by water and is protected from the effect of chlorination (Francis *et al*., 1999). It can survive temperatures of up to 67°C, undergoing cellular changes when exposed to elevated temperatures and so develop heat resistance (Farber *et al*., 1998).

# CHAPTER 3 RESEARCH

## 3.1. INTRODUCTION

The research chapter is presented in the format of scientific articles.

The objective of this study was to investigate the survival of *L. innocua* as surrogate organism to *L. monocytogenes* (O'Bryan *et al*., 2006), on broccoli after contamination of the crop through irrigation water. Firstly, the growth and survival of the pathogen was monitored on the crop in a field trial with simultaneous monitoring of reigning environmental parameters. Thereafter, the effect of minimal processing, including washing and refrigerated storage in modified atmosphere packaging, followed by heat treatment, was determined.

**Methodology used**

The methods used to enumerate and identify different microorganisms present in the water and on the produce are provided in Table 3.

**Table 3** Methodology for the detection of organisms in water and on produce

| Organism | Standard method | Media | Supplier |
|---|---|---|---|
| Total Colony Count | SABS 4833 | Plate Count Agar | Biolab, Merck, Wadeville, South Africa |
| | | Bacteriological Agar | Biolab, , Merck, Wadeville, South Africa |
| Coliforms, faecal coliforms and *E. coli* | MFHPB-19 | Lauryl Sulfate Tryptose broth<br>Brilliant Green lactose 2% Bile broth<br>EC broth<br>EMB agar | Biolab, Merck, Wadeville, South Africa |
| *Listeria* | SABS 11290-1 | *Listeria* Selective Agar Base (CM 0856) (Oxford formulation)<br>*Listeria* Selective Supplement, (SR0140E)<br>Chromogenic *Listeria*Agar Base (CM1080)<br>Chromogenic *Listeria* Selective Supplement (SR0227E) | Oxoid Ltd., Basingstone, Hampshire, England |

**Experimental design**

The experimental procedures for Phases 1 and 2 of the study were planned and executed according to the designs is Fig. 5 and Fig. 6 for Phase 1 and Phase 2, respectively.

PHASE 1

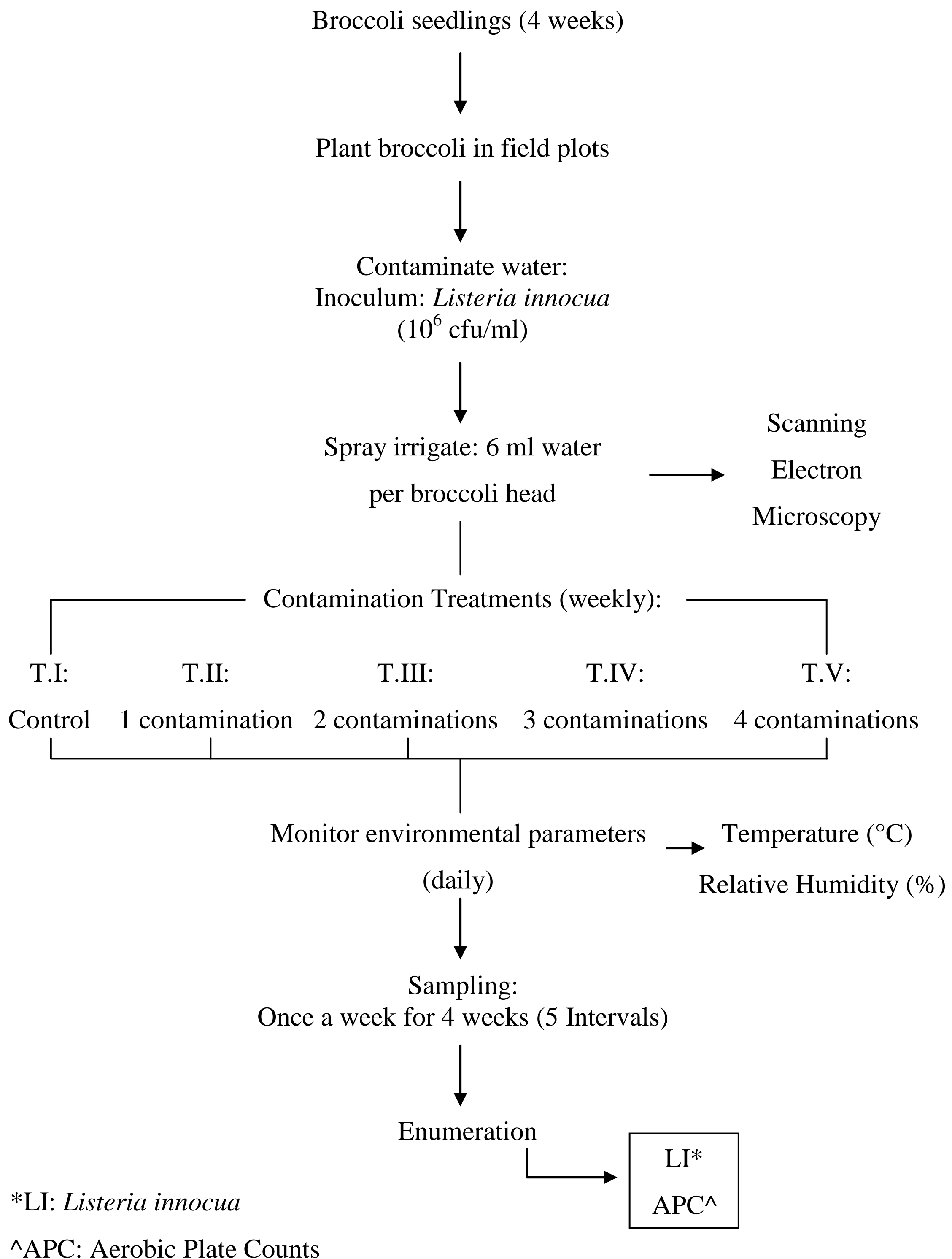
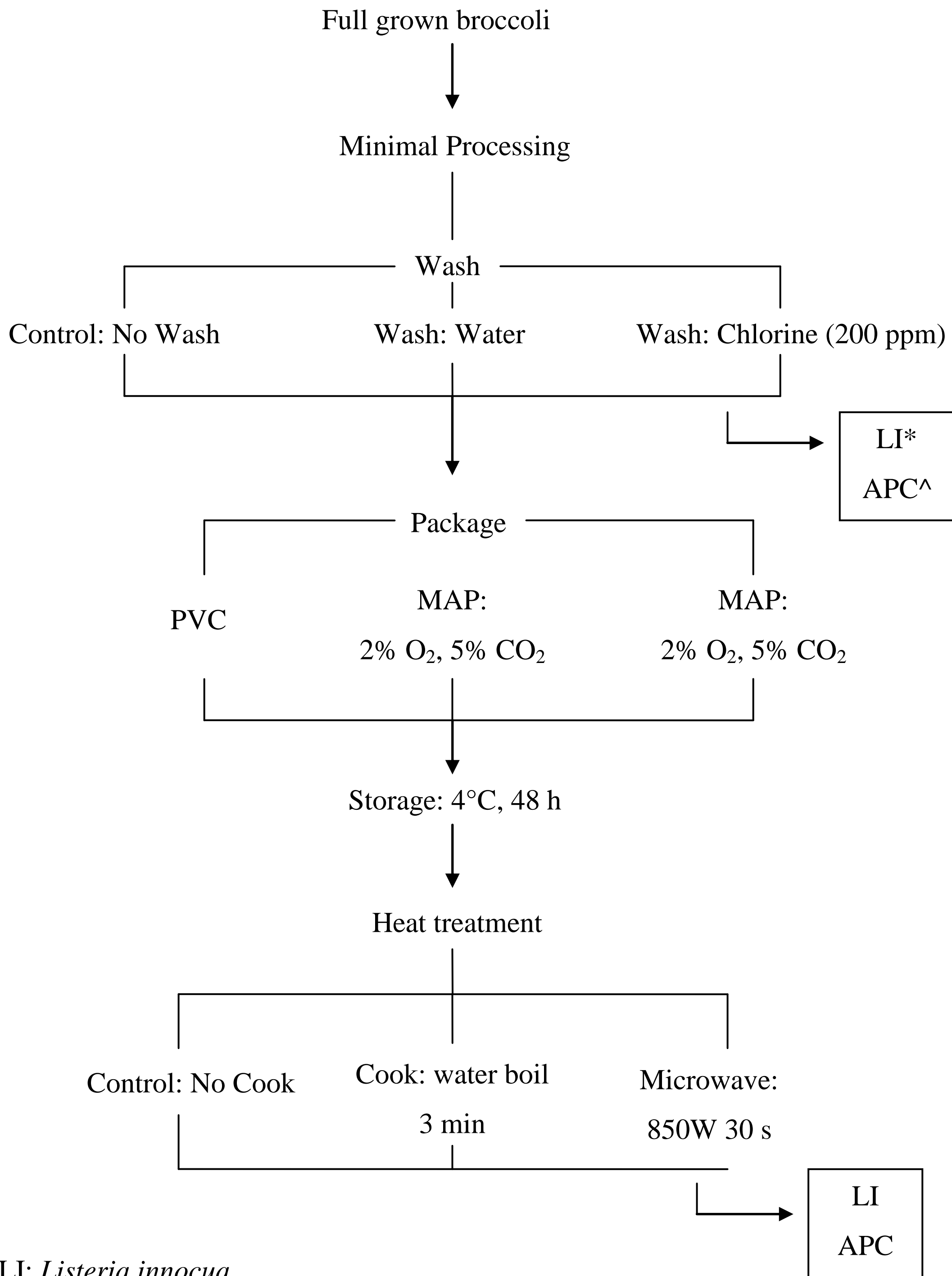



**Figure 5** Experimental design for the determination of the effect of irrigation intervals on the survival of *L. monocytogenes* on spray irrigated broccoli

PHASE 2




*LI: *Listeria innocua*

^APC: Aerobic Plate Counts

**Figure 6** Experimental design for the determination of the effect minimal processing and cooking on the survival of *L. monocytogenes* on spray irrigated broccoli

## 3.2. PHASE 1: THE EFFECT OF IRRIGATION INTERVALS ON THE SURIVIVAL OF *LISTERIA INNOCUA* AS SURROGATE TO *L. MONOCYTOGENES* ON SPRAY IRRIGATED BROCCOLI

**Abstract**

The objective of this study was to determine the survival and growth over time of *L. innocua,* as surrogate organism to *L. monocytogenes,* on broccoli grown under field conditions after application of contaminated irrigation water at predetermined intervals. *L. innocua* numbers remained similar over intervals that received consecutive inoculations and *L. innocua* numbers decreased by at least 2.3 log cfu/g after inoculation ceased, which showed an inoculation effect and that time had an influence on organism survival. Cessation of irrigation before harvest was found to effectively reduce pathogen contamination levels on the crop, whilst repeated irrigation with contaminated water contributes to maintenance of *L. innocua* as well as elevated total microbial counts on the broccoli. A lack of significant correlation between the *L. innocua* counts and the recorded environmental temperatures in the field suggested that survival is not solely dependent on and influenced by, nor can it be predicted by these parameters. It was found that the presence of high levels of contamination in irrigation water used for vegetable crops, can be associated with an increased microbial population on the crop surface.

*Keywords*: irrigation interval, *L. monocytogenes*, *L. innocua*, broccoli

### *3.2.1. Introduction*

Irrigation water in South Africa is commonly sourced from large reservoirs, farm dams, rivers, ground water, municipal supplies and industrial effluent (Britz *et al.*, 2007). Irrigation with contaminated water is one of the most prominent ways that fruit and vegetables can become contaminated with foodborne pathogens (Steele & Odumeru, 2004). As domestic foodstuffs and a very large percentage of agricultural exports are derived from irrigated lands, this shows the need for the provision of water of high quality (Barnes, 2003).

The availability of ready-to-eat fresh items produce is on the increase (Beuchat, 1996) and awareness is growing that such produce can be sources of pathogenic bacteria (Steele & Odumeru, 2004).

*L. monocytogenes* has been found on broccoli sampled from fields (Gorski *et al.*, 2003). This pathogen is widely distributed throughout the environment and its ubiquitous nature allows it to survive for long periods under adverse environmental conditions (Milillo *et al.*, 2008). It has, for example, been known to survive in plant materials for up to 10 to 12 years (Beuchat, 1996). *L. monocytogenes* causes listeriosis in humans and, although disease incidence is less frequent than other food-borne diseases, listeriosis often leads to severe consequences (Buchrieser *et al.*, 2003)

Broccoli (*Brassica oleracea* var *botrytis)* is a member of the cabbage family, grown in the Highveld region of South Africa in the winter months with a water requirement of between 30 and 38 mm of water per week. The vegetable's rough surface and the presence of crevices in the structure which causes water to be retained, aid in attachment of organisms to the crop, making this vegetable a possible substrate for organisms such as *L. monocytogenes* (Stine *et al.*, 2005).

*L. innocua* is an organism found widely and naturally in the environment, including in soil. It is closely related to the food-borne pathogen *L. monocytogenes* (Buchrieser *et al.*, 2003), but is non-pathogenic in character (Girardin *et al.*, 2005) and is thus often used as surrogate organism to study *L. monocytogenes* (O'Bryan *et al.,* 2006).

Numerous studies have examined the prevalence of pathogens in irrigation waters (Steele & Odumeru, 2004, Christison *et al.*, 2008, Taormina & Beuchat, 2001). Lötter. (2010) assessed the microbial loads present in rivers used for irrigation of

vegetables in the Western Cape, South Africa. Their study included monitoring of aerobic colony counts, enumeration of total coliforms, faecal coliforms, staphylococci, enterococci, and aerobic and anaerobic sporeformers present in the water samples. The presence or absence of the potential pathogens like *E. coli*, *Listeria* and *Salmonella*, was also determined. They concluded that the rivers investigated during their study contained high levels of contamination and that their results suggest a carry-over of pathogens from the river water to the irrigated produce.

This study therefore used *L. inoocua* as a surrogate organism to determine whether the intervals and frequency at which produce is irrigated with contaminated water before harvest affect final counts of *L. monocytogenes* on broccoli crops and was aimed at determining the effects of environmental parameters on the survival of the pathogen on the produce in the field.

### *3.2.2. Materials and Methods*

The water from the borehole with which the broccoli was irrigated daily, was sampled once every 14 days and analysed for the presence of *Listeria* spp., coliforms, *E. coli* and aerobic plate counts. Initial weekly analyses did not result in detection of any counts, and the analysis was only continued fortnightly as an additional monitoring procedure. This water was not inoculated and thus simulated a field situation where crops are irrigated daily, but possibly only with contaminated water once every other day, due to fluctuating levels of contamination in rivers as irrigation water sources.

#### 3.2.2.1. Growth, isolation and maintenance of *Listeria innocua* culture

##### 3.2.2.1.1. *L. innocua as surrogate organism for L. monocytogenes*

The studies were conducted under field trial conditions. Because of practical field and laboratory constraints, the use of *L. monocytogenes* was rendered impossible, as risk of contamination of the agricultural environment surrounding the experimental area existed. *L. innocua* was therefore used as surrogate organism during the experimental study. Even though conclusions are made and assumed to be applicable

to the pathogenic organism, the possibility does exist that differences in behaviour between the organisms could occur.

3.2.2.1.2. *Bacterial inoculum preparation*

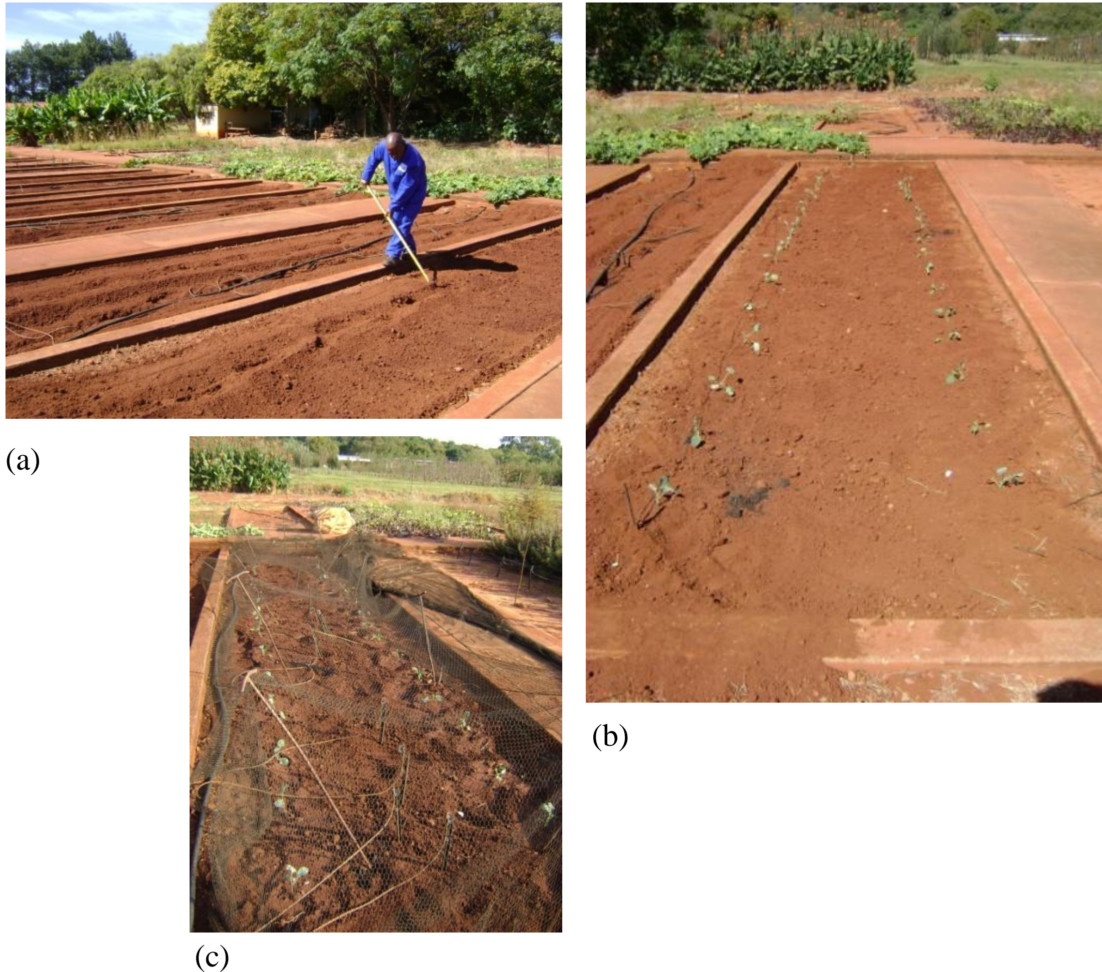
A *L. innocua* (serotype 6a, which has been fully sequenced (Nelson *et al*., 2004)) strain ATCC 33090 culture was obtained from Microbiologics (Minnesota, USA). The culture was streaked onto Tryptic Soy Agar and single colonies were isolated after 48 h of incubation at 37°C.

A single colony was inoculated into 10 ml Tryptic Soy Broth (Biolab, Merck, South Africa), which was incubated in a shaking water bath (166 rpm) at 37°C for 18 h to 20 h, a time at which the culture was at a state of transition between the late logarithmic and early stationary phase of growth (Taormina & Beuchat, 2001). The bacterial cells were harvested by dispensing into 2 ml Eppendorf tubes, which were centrifuged for 10 min at 4000 *g* (6600 rpm, RT150, Brake: 30) (Digicen20 centrifuge, Orto-Alresa). The supernatant was discarded and the harvested cell pellets were washed with three volumes of sterile saline solution (0.9% NaCl) before repeating centrifugation. The supernatant was removed again and the cells resuspended in saline at a cell density of $10^8$ cfu/ml, by means of comparison with the MacFarland standard (0.5) (Bhagwat, 2003). The solution was diluted further in sterile distilled water to a cell density of $10^6$ cfu/ml. These cell numbers were confirmed by enumerating on *Listeria* selective media (Oxford formulation) (Oxoid, Basingstone, UK).

3.2.2.2. Planting and growth of broccoli

Broccoli seedlings, 4 weeks of age were obtained from a local nursery in Pretoria, South Africa and transplanted onto three soil field plots, as three replicates, as schematically represented in Fig. 7, at the experimental farm of the University of Pretoria, Pretoria, South Africa. The plots were watered with 30-38 mm/m$^2$ of water from the borehole per week. Each block represented a replicate study, with five treatments, randomly assigned, within each replicate, as illustrated in Fig. 8. The three replicate studies were performed, commencing with the first treatments during

three consecutive weeks. The plots were located outside in open air and were covered with net, to protect the plants from birds. No other protective covering was erected over the plots. The plants were allowed to grow for a period of 12 weeks to allow size development of broccoli heads, before contamination and experimentation commenced (experimental layout designed using Girardin *et al*. (2005) as guideline).



(a) (b) (c)

**Figure 7** The field plots at the experimental farm; (a) preparing the soil; (b) the planted seedlings; (c) protective net covering

Broccoli is a crop susceptible to white rot, making it difficult to grow under organic conditions. Extra crops were planted in each plot, to make provision for losses due to rot or other crop growth problems. Crops affected by such phenomena were not used for the experimental analysis and did thus not affect of cause variation in data.

| **Treatment** | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **I** | | | | **III** | | | | **V** | | | **IV** | | | **II** | | | |
| **1** | **2** | **3** | **4** | **1** | **2** | **3** | **4** | **1** | **2** | **3** | **1** | **2** | **3** | **1** | **2** | **3** | **4** |
| **1** | **5** | **(5)** | **(5)** | **5** | **(5)** | **(5)** | **4** | **5** | **(5)** | **(5)** | **4** | **5** | **(5)** | **(5)** | **5** | **(5)** | **(5)** |

(a) Replicate 1

| **Treatment** | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **IV** | | | **III** | | | | **II** | | | | **V** | | | **I** | | | |
| **1** | **2** | **3** | **1** | **2** | **3** | **4** | **1** | **2** | **3** | **4** | **1** | **2** | **3** | **1** | **2** | **3** | **4** |
| **4** | **5** | **(5)** | **(5)** | **5** | **(5)** | **(5)** | **5** | **(5)** | **(5)** | **4** | **5** | **(5)** | **(5)** | **1** | **5** | **(5)** | **(5)** |

(b) Replicate 2

| **Treatment** | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **III** | | | **V** | | | | **I** | | | | **II** | | | | **IV** | | |
| **1** | **2** | **3** | **1** | **2** | **3** | **4** | **1** | **2** | **3** | **4** | **1** | **2** | **3** | **4** | **1** | **2** | **3** |
| **4** | **5** | **(5)** | **(5)** | **5** | **5** | **(5)** | **1** | **5** | **(5)** | **(5)** | **5** | **(5)** | **(5)** | **4** | **5** | **(5)** | **(5)** |

(c) Replicate 3

**Figure 8** Sampling layout in field plots according to a randomised block design, for replicate 1 (a), 2 (b) and 3 (c). Five treatments (coded in white (I), yellow (II), green (III), blue (IV) and red (V) were split into 7 blocks for the 5 intervals within each treatment (1 – 5), with two crops extra per treatment in case of occurrence of crop damage (5)

### 3.2.2.3. Contamination of broccoli plants

The method of irrigation can influence how effectively pathogens present in irrigation water are transmitted to plant surfaces. During spray irrigation, the edible portions of the plant are wetted directly (Steele & Odumeru, 2004). This is especially the case with a vegetable crop such as broccoli, which is not protected by an outer layer of

leaves that are removed before eating (Orzolek *et al*., 2000). This method of irrigation is thus expected to have an increased risk of pathogen transfer in comparison to, for example, drip irrigation, due to the large degree of contact with the product surface, motivating the use of this irrigation method in the study. Facilitating effective and lengthy contact of the irrigation water with the broccoli surface proved to be difficult due to the hydrophobic nature of the crop surface. The use of a surfactant in the application fluid could improve this contact and thus access of the pathogen to the surface, thus simplifying experimental conditions and lead to more definitive results in terms of cell numbers. It was, however decided against the use of a surfactant as this would not simulate actual field conditions.

Sterile distilled water inoculated with *L. innocua* at a cell density of $10^6$ cfu/ml, was applied to the grown broccoli plants (seedlings of four weeks planted and allowed to grow in the field plot for another twelve weeks) by means of a sterile 10 ml syringe. The syringe was held at an approximate distance of 20 cm above the head of the broccoli plant and 6 ml of the inoculated water was applied to each plant. The water was applied specifically to the part of the plant that was likely to be consumed. The water was applied drop-wise, to simulate dropping of the water onto the plant surface during spray irrigation.

The plants were inoculated as shown in the treatment schedule in Table 4, over a period of four weeks. The weekly designated samples were treated with contaminated water on the first day of the week, i.e. days 1, 8, 15, and 22. Samples were also taken on the same days with day 29 representing the final sampling occasion for treatment V, seven days after inoculation. These inoculation and sampling occasions will be referred to as intervals 1 through 5, with interval 5 representing a sampling occasion only. Treatment I therefore represents the control and only uncontaminated water was applied to these crops, whilst the crops in Treatment V were treated weekly with inoculated water on the first four inoculation occasions, or intervals. The different treatments serve to monitor the survival of the applied organisms and to make the data obtained statistically comparable.

Broccoli crops were irrigated daily with uncontaminated borehole water, but only received treatments for purposes of the experiment with inoculated water once a week.

**Table 4** Inoculation schedule over 4 weeks (5 Intervals)

| **Treatment** | **Number of inoculations** | **Interval** | | | | |
|---|---|---|---|---|---|---|
| | | **I1** | **I2** | **I3** | **I4** | **I5** |
| TI | **0** | ○ | ○ | ○ | ○ | ○ |
| TII | 1 | ● | ○ | ○ | ○ | ○ |
| TIII | 2 | ● | ● | ○ | ○ | ○ |
| TIV | 3 | ● | ● | ● | ○ | ○ |
| TV | 4 | ● | ● | ● | ● | ○ |

● Application of inoculated water

○Application of uninoculated water

Sterilized, distilled water was used for the inoculation, to facilitate greater control of additional variables in the experimental analysis. However, as the distilled water could have caused a certain loss in viability, using sterilized borehole water would have been a more suitable alternative.

### 3.2.2.4. Sampling of broccoli plants

Sampling occurred once a week. Crops were contaminated between 08h00 and 10h00 and samples were taken by hand harvesting 30 min after application of the treatment (contaminated water or uncontaminated water as control treatment). Two samples per treatment were taken for analysis. The head of the broccoli was sampled, by cutting the broccoli at the stem below the head on which the inoculum was applied. The sampled broccoli heads were placed into sterile plastic stomacher bags for transport to the laboratory. Two samples from each treatment were taken on a weekly basis on the sampling occasion or interval, seven days after inoculation so as to monitor bacterial growth or survival over seven days. Broccoli heads were trimmed to 15 cm total length and had a final weight of 10g.

Broccoli samples were stomached for 2 min in 90 ml Buffered Peptone Water (0.1%) (Merck), from which serial dilutions were prepared and spread plated onto *Listeria* Selective media (Oxford formulation) (Oxoid) for enumeration. Duplicate samples for each treatment was taken, with each of the samples being analysed in

triplicate. The plates were incubated at 37°C for 24 to 48h to allow enumeration of *L. innocua* colonies. Typical colonies were confirmed on Chromogenic *Listeria* Agar Base medium (Oxoid).

*Listeria* spp., are incubated on Oxford Listeria Agar at 37°C for 24h to 48h. During this study, more colonies were observed after an incubation period of 48h, than after 24h and all plates were subsequently analysed after two days' incubation.

PALCAM *Listeria* Selective Agar Base is an alternative media to Oxford for the detection and isolation of *L. monocytogenes*. Oxford media was chosen due to the greater simplicity of the enumerative conditions. Simultaneous enumeration of samples on both media would provide confirmation of counts and more accurate results of cell numbers under aerobic as well as anaerobic growth conditions. This would be valuable for a facultative anaerobe such as *L. monocytogenes*.

FRASER *Listeria* Selective enrichment Broth and Supplement for the selective enrichment of *Listeria* creates optimum growth conditions for *Listeria* due to the high nutrient content and the large buffer capacity (Fraser & Sperber, 1988). During initial sample enumerations in the first field trial replicate, samples that tested negative on Oxford media were further enriched in FRASER medium, but negative results were repeatedly obtained, so this procedure was not followed in further analyses.

Plate Count Agar medium is generally used for the determination of the total viable microbial contents in food samples, as it is free from any inhibitory ingredients or indicators. It was used in this analysis to determine the aerobic plate counts on the broccoli. The samples were surface plated, which provided the benefit that plates could be pre-poured, stored and moved around easily. The PCA was incubated at 25˚C to enable growth of both psychrotrophs and mesophiles present on broccoli for representative enumeration (SANS 4833; ICMSF, 1978).

As not all organisms are transferred from the inoculation water onto the broccoli heads, the hydrophobicity of the broccoli surface casing the water to run off easily, initial counts for analyses were not taken as the inoculation level in the contamination water, but as the count on the crop after first sampling (30 min after inoculation, as described above).

### 3.2.2.5. Monitoring of environmental parameters

The environmental parameters to which the broccoli plants were naturally exposed in the field were monitored daily and recorded weekly at the sampling intervals. These environmental parameters included minimum and maximum temperatures, relative humidity as well as rainfall. The data was recorded in the Meteorological Diary of the Climatological Station (no. 0513465_1), situated at the University of Pretoria Experimental Farm.

### 3.2.2.6. Statistical analysis

#### 3.2.2.6.1. *Analysis of Variance*

All the samples used for colony enumeration, including controls were analysed in duplicate. The mean values of triplicate plate counts of duplicate samples were calculated and reported with 95% confidence interval. Data was subjected to analysis of variance (ANOVA) (Han *et al*., 2000) and it was determined if significant differences ($P< 0.05$) exist between mean values.

The experiment was designed as a randomised complete block design (RCBD) with four *L. innocua* treatments and a control treatment (no contamination) randomly assigned to plots within 3 blocks. Eight plants were established per plot. One broccoli plant per plot and per block within each plot area was inoculated weekly and the number of organisms determined by sampling 30min after application.

Factorial analysis of variance (ANOVA) was used to test for differences between the treatments, weeks and treatment by week interaction. The counts were log (base 10) transformed to normalise the data. The treatment variances were, however, still not homogeneous after transformation. Glass *et al*., (1972) indicated that the consequences on inference after ANOVA are serious when a transformation does not rectify the problem of heterogeneous variances and recommend testing at a stricter level of significance. Means were thus separated using Fishers' protected t-test least significant difference (LSD) at the 1% level, instead of the accepted 5% level, of significance (Snedecor & Cochran, 1980).

The resulting data was used to draw a correlation between the environmental conditions and the growth of *L. monocytogenes* on broccoli.

3.2.2.6.2. *Regression Analysis*

The calculated correlation coefficients, also known as Pearson's coefficient of correlation or the product moment correlation coefficient, is a measure of the linear relationship between two random variates ($-1 < r < 1$). Note that this only shows the extent to which two variates are linearly related and does not imply any causal relationship between them (Kleinbaum *et al*., 2008).

Generally, a coefficient of about ±0.7 or more is regarded as indicating fairly strong correlation, and in the region of ±0.9 it indicates very strong correlation. In the region of ±0.5 the correlation is moderate, and in the range –0.3 to +0.3 it is weak (Rayner, 1969). For example, if $r = 0.5$, even if statistically significant, the $R^2$ = 25%. This indicates that 25% of the variation between the observations is accounted for by the relationship between the two variates, but 75% variation remains unexplained. The regression data only shows the extent to which two variates were linearly related and does not imply any causal relationship between them (Kleinbaum *et al*., 2008, M. Smith, personal communication, 2009).

All data were analysed using the statistical programme GenStat® (2007) (Payne *et al*., 2007).

3.2.2.7. Microscopy

The microscopy study was performed with the aim of gaining insight into the attachment of the pathogen to the broccoli surface, including the method of attachment, in addition to supporting the microbial counts determined from plating. It has been shown that microorganisms attached to the surfaces of fruits and vegetables displayed increased resistance to sanitation treatments (Han *et al*., 2000).

Confocal Laser Scanning Microscopy was the original method of choice for the microscopy study. This technique has been used extensively in food microbiology studies in investigations of location and viability of microorganisms in food products. CLSM provides the advantage of information about microbial viability and identity in the form of three-dimensional images (Han *et al*., 2000). For microbial visualisation with CLSM, an FITC antibody to the pathogen is needed to label the bacteria on the

surface. The FITC-Ab stains all cells green, whilst dead cells are stained red by propidium iodide. This double staining results in live cells staining green and dead cells staining red, which monitors viability of cells (Seo & Frank, 1999). Failure to obtain an FITC antibody to *L. monocytogenes* as well as the high cost of this specimen, forced the use of the LIVE/DEAD BacLight kit, staining with thiazole orange (TO) and propidium iodide (PI). This method produced unsatisfactory results, as staining was not successful to differentiate between cells and great depth of study and expertise in this technique of microscopy was needed. It was therefore decided to revert to the more tried and tested technique of Scanning Electron Microscopy, which is often used to study the colonisation and attachment of microbes to food (Han *et al*., 2000). This technique reveals information about the surface details of cells.

3.2.2.7.1. *Scanning Electron Microscopy*

Broccoli florets (4 mm x 1 mm) were suspended in inoculated water (2 x $10^7$ cfu/ml) for 30 min to allow *L. innocua* organisms to attach to the surface. The inoculated florets were fixed overnight in 2.5% gluteraldehyde and rinsed three times with 0.075 M sodium phosphate buffer at pH 7.0. The florets were further fixed in 2% osmium tetroxide for 1h and rinsed again three times with 0.075 M sodium phosphate buffer. Fixed samples were dehydrated in a graded ethanol series (50%, 70% and three times 100%), at room temperature. The samples were critical-point dried for three hours and mounted on specimen stubs before being sputter-coated with a 30 nm layer of gold-palladium. The surfaces of the florets were then examined with a scanning electron microscope (JEOL JSB 840) for bacterial attachment (Arnold & Bailey, 2000; Pathan *et al*, 2008; A. Hall, personal communication, 2010).

### *3.2.3. Results*

During the five week monitoring period a maximum of 20.9°C was reached during the day and the lowest temperature recorded during the night was 2.4°C, whilst an average relative humidity of 73.9% was maintained during the growth period of the crops. The water that was applied to the crops was inoculated with an average level of 6.4 log cfu/ml *L. innocua* (Table 5).

**Table 5** Environmental parameters measured at the University of Pretoria experimental farm and the contamination level of *L. innocua* in the water at each irrigation interval

| Interval | Environmental parameter (weekly average) | | | Water inoculation level (log cfu/ml) |
|---|---|---|---|---|
| | Temperature (°C) | | Relative Humidity (%) | |
| | Maximum | Minimum | (RH) | |
| 1 | 15.6 | 2.4 | 70.4 | 5.9 |
| 2 | 16.4 | 4.7 | 73.7 | 6.5 |
| 3 | 20.9 | 7.7 | 77.4 | 6.5 |
| 4 | 20.4 | 9.2 | 79.0 | 6.6 |
| 5 | 20.1 | 7.1 | 69.1 | 0 |

#### 3.2.3.1. Survival of *L. innocua* and aerobic plate count for the inoculation treatments over five intervals

Mean values of *L. innocua* and aerobic plate count (APC) on the broccoli grown in the field for the treatments (T) and intervals (I) were determined by means of Analysis of Variance. Treatment I (TI) was the uninoculated control, Treatment II (TII) administered 1 inoculation at Interval 1 (I1) and Treatment V (TV) 4 inoculations across the first four Intervals, with no inoculation taking place at Interval 5 (Table 6).

**Table 6** Mean *L. innocua* levels and Aerobic plate count on broccoli grown in the field over 4 weeks, treated and sampled at 5 intervals

| | **Treatment (T)** | | **Interval (I)** | |
|---|---|---|---|---|
| | ***L. innocua*** | **Aerobic plate count** | ***L. innocua*** | **Aerobic plate count** |
| | | **log cfu/g** | | |
| TI, I1 | 0.56[c] | 3.15 | 2.56[a] | 3.83[a] |
| TII, I2 | 0.98[c] | 3.22 | 2.44[a] | 3.67[ab] |
| TIII, I3 | 1.52[bc] | 3.33 | 1.66[ab] | 3.5[abc] |
| TIV, I4 | 2.39[ab] | 3.435 | 1.18[bc] | 3.12[bc] |
| TV, I5 | 2.78[a] | 3.966 | 0.40[c] | 2.99[c] |
| SEM* | 0.31 | 0.17 | 0.31 | 0.17 |
| P* | <.001 | 0.012 | <.001 | 0.004 |
| LSD(1%) | 1.16 | 0.64 | 1.16 | 0.64 |

* SEM: Standard error of means
P: Probability
LSD: Fischer's Least significant difference

Values in the same column followed by the same superscript, are not significantly different from each other. Fischer's protected LSD for the treatment means for aerobic plate count was not calculated as the variance ratio was not significant (M. Smith, personal communication, 2009)

3.2.3.1.1. *Main effects of Treatments and Intervals on bacterial counts*

For the *L. innocua* counts across treatments, the mean values for TV and TIV were significantly higher than TI, and TII. The aerobic plate count also increased marginally, although the differences were not significant ($P > 0.001$) (Table 6)

The mean value of *L. innocua* detected across the treatments at I1, was significantly higher than at I4 and I5 (Table 6).

3.2.3.1.2. *L. innocua counts on broccoli for inoculation treatments over 5 intervals*

The *L. innocua* counts for the control treatment were similar over 5 intervals, with the natural *Listeria* population initially being 1.57 log cfu/g and finally dying off completely (Fig. 9). For TII (1 inoculation), I1 differed significantly ($P < 0.01$) from I4. No survival was detected at I4 (3 weeks after the last inoculation), but *L. innocua*

was detected again at a low level of 0.9 log cfu/g at I5 (after 4 weeks). The broccoli crops in TIII received 2 inoculations with *L. innocua*, for which I1 differed significantly from I3 ($P$< 0.01), whilst I2 differed significantly from I3, I4 and I5 ($P$< 0.01). TIV facilitated the administration of three inoculations and the counts at I2 and I3 differed significantly from those in I5 ($P$< 0.01). The *L. innocua* numbers detected for TV (4 inoculations) indicated that I1, I2, I3 and I4 were significantly higher than I5 ($P$< 0.01), at which no survival was detected, with the counts over the first four intervals being similar, at a level of between 2.91 and 3.86 log cfu/g (fig. 9).

*L. innocua* numbers decreased significantly ($P$< 0.01) to less than 1.4 log cfu/g (and by at least 2.3 log cfu/g) 7 days after the last inoculation in each treatment. The numbers decreased to less than 0.9 log cfu/ml, 4 weeks after first exposure on the crop, both in treatments with single and accumulative contaminations (fig. 9).

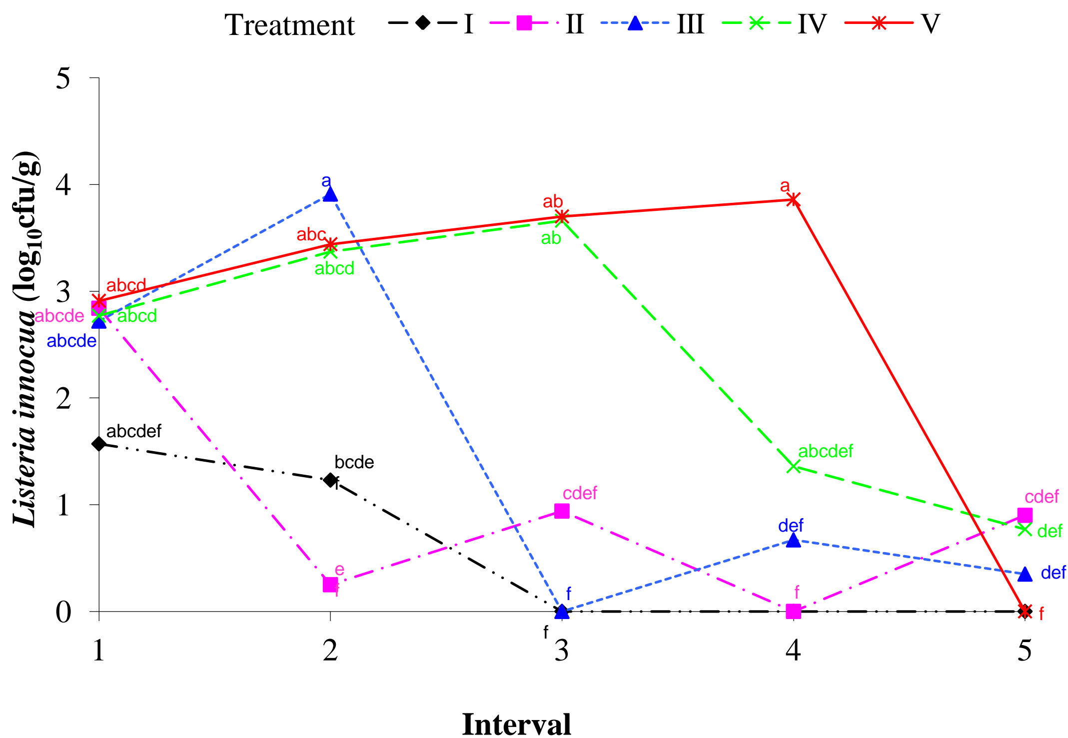



**Figure 9** The effect of time and inoculation frequency on *L. innocua* counts on broccoli over 5 weekly intervals (SEM 0.68, P = 0.006, LSD(1%) 2.59, CV 71.8%). Interval (I) = 7 days. Intervals inoculated: Treatment TI (control): 0; TII: I1; TIII: I1, I2; TIV: I1, I2, I3; TV: I1, I2, I3, I4. Points on the graph with the same letter are not significantly different from each other ($P$ >0.01)

3.2.3.1.3. *Bacterial level on broccoli for inoculation treatments over 5 intervals*

The APC for TI remained at an average level of 3.15 log cfu/g over all 5 intervals, as illustrated by Fig. 10). A significant decrease ($P< 0.01$) in the APC, from 4.71 log cfu/g to 2.59 cfu/g was observed for TII from the last inoculation at I1 to the consecutive interval. The counts thereafter (I2, I3, I4 and I5) remained similar. A significant decrease also occurred during TIII ($P< 0.01$), with numbers dropping from 4.46 log cfu/g at I2 to 2.59 log cfu/g at I3 and the counts remaining similar over the following intervals. The APC for TIV remained similar ($P>0.01$) over the intervals, but the mean count for I4 and I5 was more than 1 log unit lower than the mean of I1, I2 and I3. Counts over inoculated intervals in TV were similar before a significant decrease ($P< 0.01$) was again observed in T5 between I4 (the last inoculation, 4.47 log cfu/g) and I5 (3.15 log cfu/g). All final microbial counts (at I5) were between 2.7 and 3.4 log cfu/g, a level which was not significantly different from initial counts which were at 2.95 log cfu/g (Fig.10).

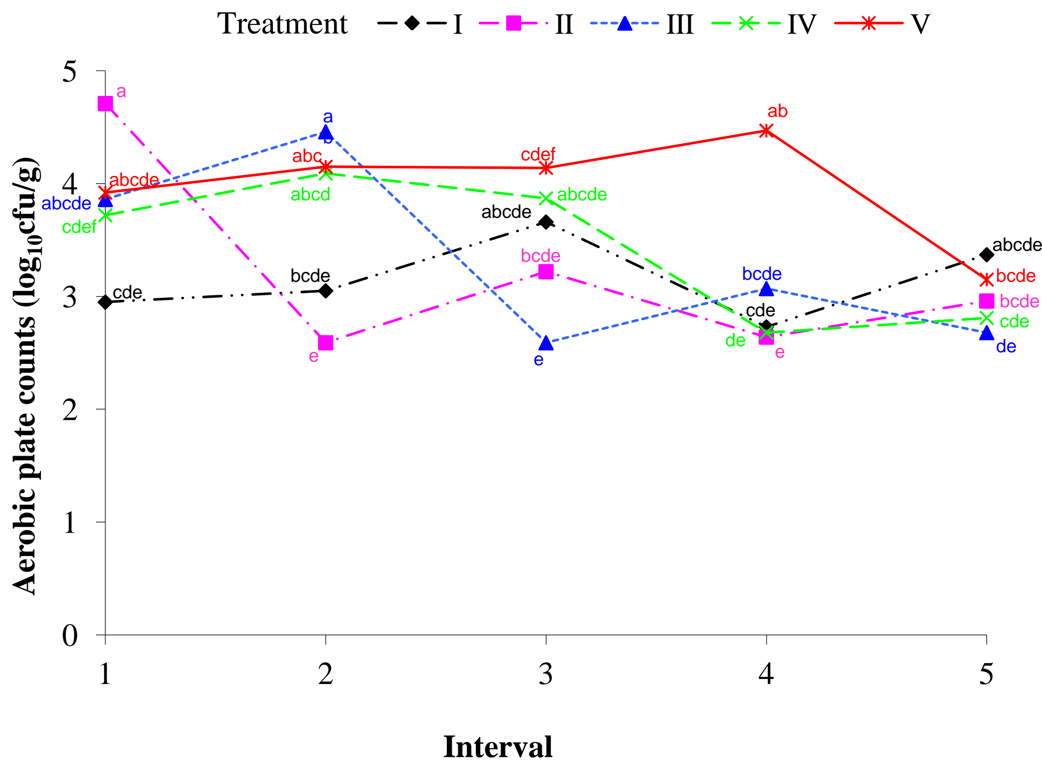



**Figure 10** Effect of time and inoculation frequency on aerobic plate counts on broccoli over time (SEM 0.38, P = 0.006, LSD(1%) 1.44, CV 19.2%). Interval = 7 days. Intervals inoculated: Treatment TI (control): 0; TII: 1; TIII: 1, 2; TIV: 1, 2, 3;

TV: 1, 2, 3, 4. Points on the graph with the same letter are not significantly different from each other ($P$ >0.01)

### 3.2.3.2. Regression Analysis

The regression is discussed where an r ≈ 0.7 (fairly strong), or r ≈ 0.5 (moderate) was obtained (Table 7).

**Table 7** Summary of regression data to illustrate the correlation between *L. innocua*, aerobic plate count and environmental parameters

| T | Parameters | Correlation | r | Probability | % Variance |
|---|---|---|---|---|---|
| I | Temp_Min + LI | Fairly strong | -0.669 | 0.0062 | 40.4 |
| | Temp_Max + LI | Moderate | -0.529 | 0.0394 | 22.4 |
| II | **APC + LI** | Fairly strong | 0.667 | 0.00641 | |
| | Temp_Min + LI | Moderate | -0.472 | 0.06701 | 16.3 |
| | Temp_Min + APC | Moderate | -0.470 | 0.06835 | 3.0 |
| III | **APC + LI** | Fairly strong | 0.862 | 0.00003 | |
| IV | **APC + LI** | Fairly strong | 0.814 | 0.00019 | |
| | Temp_Max + LI | Moderate | -0.504 | 0.05015 | 19.7 |
| | Temp_Max + APC | Moderate | -0.592 | 0.01913 | 30.0 |
| | Temp_Min + APC | Moderate | -0.508 | 0.04858 | 20.1 |
| V | **APC + LI** | Fairly strong | 0.871 | <0.001 | |
| | RH% + LIS | Moderate | 0.607 | 0.0156 | 32.0 |
| | RH% + APC | Moderate | -0.610 | 0.0150 | 32.4 |

T: Treatment

% Variance: % Variance accounted for by the parameters

LI: *Listeria innocua*

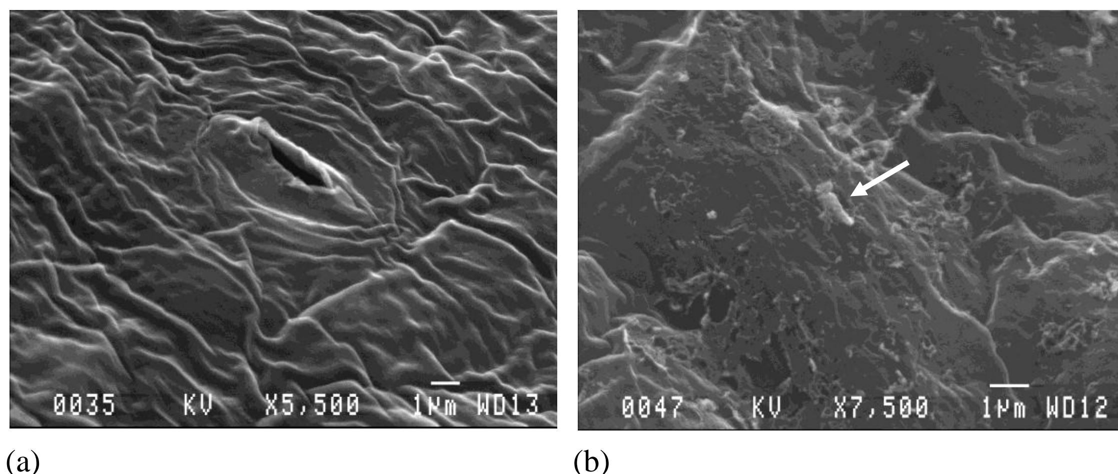
APC: Aerobic plate count

Parameters not indicated in Table 7 had corresponding r-values that only indicated a weak correlation between the microbial counts and the environmental parameters.

None of the parameters exhibited very strong (r ≈ 0.9) correlations. In all four treatments that included *L. innocua* contamination (TII, TIII, TIV, and TV), a fairly strong correlation was observed between the *L. innocua* counts and the aerobic plate counts (r ≈ 0.7), with this correlation becoming slightly stronger with treatments that received a greater amount of inoculations over consecutive intervals. A moderate correlation between minimum temperature and *L. innocua* counts was observed, and a moderate correlation between maximum temperature and *L. innocua* counts was also noted in two treatments.

### 3.2.3.3. Microscopy

The surface of the broccoli floret was visualised under the scanning electron microscope (JEOL JSB 840). Rod-shaped bacterial cells could be visualised (Fig. 11).




(a) (b)

**Figure 11** The surface of the broccoli floret as viewed under scanning electron microscopy; a) the smooth surface and stomata; b) the surface with possible bacterial adhesion

### *3.2.4. Discussion*

The mean values for *L. innocua* increased across treatments because of an increasing number of consecutive weekly inoculations. Mean values decreased over intervals, due to fewer inoculations for all treatments over time.

Under conditions of no treatment, *L. innocua* was initially detected on the crop (from TI, the control treatment) suggesting the possibility of survival of this population on the crop during crop growth. In a recent study (Ijabadeniyi & Buys, 2011), *L. monocytogenes* was isolated from broccoli grown in the field. The *L. innocua* levels correlated with the total microbial counts on the crop, also remaining fairly constant during the growth period, before any treatments and after final treatment ceased Beuchat, (2002b) also referred to the detection of autochtonous microbial populations on vegetables grown in the field.

In all treatments it was observed that numbers of *L. innocua* remained fairly constant over intervals that received consecutive inoculations, and *L. innocua* numbers decreased significantly after inoculation ceased. This showed an inoculation effect and that time had a significant effect on organism survival. The background flora (APC) behaved in this same way, with cell counts decreasing after the last inoculation in each treatment. As in the case of most studies facilitating contamination through laboratory inoculation, the *L. innocua* concentration in this study was high and at levels greater than would naturally occur on produce in the field. The background flora could therefore have been comprised largely of the *L. innocua* inoculated onto the produce, contributing to the similar trend in levels of the pathogen and the aerobic plate counts. The high inoculum level can also explain the observed lack of competition between the pathogen and the natural microflora. Schoeller *et al*. (2002), suggested that the microflora on a crop surface could impede the establishment and growth of *L. monocytogenes*. Schuenzel & Harrison (2002), also found the natural microflora of minimally processed produce to be inhibitory to *L. monocytogenes*. The results of Ongeng *et al*. (2007), however also showed that background bacterial flora did not have an effect on the growth of *L. monocytogenes* on vegetables.

Keraita *et al*. (2007), found that cessation of irrigation before harvest of vegetables effectively reduced microbial contamination. Their results also showed

that the final levels of pathogens on lettuce depended on the initial contamination levels. Dreux *et al*. (2007), concluded that if the surface of parsley, which has a surface similar in hydrophobicity to broccoli, were to be directly contaminated with *L. monocytogenes*, for example through contaminated irrigation water, the resultant product for consumption would not be contaminated unless contamination occurred a very short period before harvest. As farmers usually irrigate until the harvest day (Keraita *et al*. 2007), or harvest crops within 24 h of the last irrigation Tyrell *et al*. (2006), the risk posed by contamination is relevant. Heaton & Jones (2008), also commented that the interval between irrigation and harvest plays an influential role in the likelihood of pathogenic bacteria surviving to reach the consumer.

In Treatment III, possible growth or recovery was noticed 4 weeks after the last inoculation, or external contamination could most likely have occurred from irrigation water, as was also suggested by Girardin *et al*. (2005), and Brackett (1999b), especially from overhead irrigation. The fact that the *L. innocua* levels on the crop did not change much during over the first four intervals in TV, suggests that the periodic inoculation was responsible for the maintenance of the *L. innocua* load on the broccoli surface. The results of Lötter (2010), also indicated a possible "build-up" of contamination on produce with the repeated application of tainted irrigation water. Even though Milillo *et al*. (2008), found that over ten days of plant growth, *L. monocytogenes* numbers increased on the surface, the contribution of inoculation was, in this case, not great enough to significantly increase the numbers with continued contamination, suggesting that pathogen die-off did occur in the time between inoculations. Keraita *et al*. (2007), reported that limited field trials provided a rough estimate of 0.5–2.0 log units of pathogen reduction per day during crop growth, whilst Dreux *et al*. (2007), observed decreases in *L. monocytogenes* on the parsley surfaces in the field by several log units within 2 days after being contaminated.

Despite assumed pathogen die-off, *L. innocua* was still detected seven days after final inoculation, albeit in low numbers, which agreed with the findings of Beuchat and Brackett (1991), who showed that *L. monocytogenes* is capable of surviving on lettuce. Girardin *et al*. (2005), however, reported that *L. innocua* populations directly inoculated on parsley leaves decreased substantially within 48 h under field conditions and that no *Listeria* could be detected after 48 h. *Listeria* remaining on the broccoli surface could have belonged to a minor fraction of the initial population that was presumably more resistant to the plant surface conditions.

Results of a study by Flessa *et al*. (2005), indicated that *L. monocytogenes* displayed the ability to remain viable, but not to grow on strawberries.

The mere presence of *L. monocytogenes* on fresh vegetables is considered as contamination. The FDA in the United States (Heaton & Jones, 2008) currently enforces a zero-tolerance policy for *L. monocytogenes* in ready-to-eat foods, and even guidelines put in place by the South African Department of Health state that *L. monocytogenes* should be absent in one gram of raw vegetable produce (Lötter, 2010; Department of Health, 2006). The detection of any *L. monocytogenes* in the food therefore deems the product adulterated, classifying a food product as unsuitable for human consumption (Gandhi & Chikindas, 2007).

The correlation of *L. innocua counts* with the minimum temperature is negative. The minimum temperatures were those recorded during the night. This inverse correlation could most likely be ascribed to , organisms not surviving over time due to lack of sufficient nutrients on the plant surface, rather than lower temperatures favouring organism survival, or higher night-time temperatures preventing growth. Flessa *et al*. (2005), suggested that *L. monocytogenes* was unable to survive on intact vegetable surfaces due to the surface structure of the crop prohibiting access of the organism to adequate moisture and nutrients. Low night-time temperatures should not impede the survival of *L. monocytogenes*, as this organism has been proven to be able to survive even at refrigeration temperatures ($<$ 4°C) (Beuchat, 2002a), possibly by the process during which the fatty acid chain length is shortened. This decreases the interaction between carbon atoms of neighbouring chains in the cell membrane, resulting in the maintenance of optimum membrane fluidity needed for growth at low temperatures (Gandhi & Chikindas, 2007; Beales, 2004).

With the moderate correlation observed between the maximum temperature and *L. innocua* and on one occasion the aerobic plate counts, the negative correlation also suggested that increasing temperatures caused cell numbers to decline. This was in accordance with the findings of Keraita *et al*. (2007), who established that pathogen inactivation on crops is more rapid in hot, sunny weather than in cool, cloudy or rainy conditions. Fattal *et al*. (2002), also made the observation that in very dry and hot climatic conditions, high pathogen reduction rates in the field are expected. Cells were exposed to sunlight on the surface of the broccoli, which could have caused membrane permeabilisation to inactivate organisms (Russell, 2000). Average

maximum temperatures recorded during the day were, however, not very high (maximum 20.9°C). In addition, cells were possibly protected to a degree from these rays by the netting erected over the plants to prevent access of birds to the crops in the plot. Studies have found that pathogens are killed on vegetables if they are exposed to unfavourable climatic conditions (Shuval *et al*., 1986; Yates *et al*., 1987), taking into consideration the low percentage of variance that is accounted for (as low as 20%). Critical analysis of these maximum temperatures, however, leads one to the assumption that the behaviour of the organisms was in this case not directly attributed merely to the conditions of and change in atmospheric temperature, but more likely to the effect of time and subsequent availability of nutrients on the plant surface, as was also concluded by Flessa *et al*. (2005). The statement is also applicable to the moderate correlation observed between cell counts and relative humidity (RH) in T5, despite the observation by Dreux *et al*. (2007), that *Listeria* spp. populations declined under conditions of low RH.

Rainfall occurred on two occasions during the field trial, but no significant change in *L. innocua* numbers or APC was observed after the occurrences.

A fairly strong positive linear correlation between *L. innocua* numbers and total aerobic contamination was observed. This indicated that the presence of high levels of contamination (with, in this case *L. innocua*) in irrigation water used for vegetable crops, can be associated with an increased microbial population on the crop surface. A lack of correlation of *L. innocua* numbers with the environmental parameters (percentage variance accounted for <40%), suggested that survival is not solely dependent on and influenced by, nor can it be predicted by these parameters.

### *3.2.5. Conclusions*

Irrigation with water containing high *L. innocua* numbers results in an elevated load of the organism on broccoli immediately after irrigation, suggesting possible organism transfer from the water to the vegetable. Applying irrigation water containing high numbers of *L. monocytogenes* therefore further also contributes to an elevated total microbial load on broccoli. Continued application of such contaminated water on broccoli contributes to maintenance of *L. innocua* on broccoli. Cessation of irrigation results in significant reduction of the *L. innocua* on broccoli per-harvest. Due to the

surrogate nature of *L. innocua* to *L. monocytogenes* (O'Bryan *et al*., 2006), cessation of irrigation can be considered a possible method to reduce the burden of pathogens such as *L. monocytogenes* on the crop at harvest and subsequently at point of sale.

# 3.3. PHASE 2: THE EFFECT OF MINIMAL PROCESSING AND COOKING ON THE SURVIVAL OF *LISTERIA INNOCUA* AS SURROGATE TO *L. MONOCYTOGENES* ON SPRAY IRRIGATED BROCCOLI

**Abstract**

The aim of this study was to determine the effect of minimal processing and subsequent cooking on the survival of *L. innocua* on broccoli. Washing with water caused a 1 log reduction of *L. innocua*, whilst washing with 200 ppm chlorinated water facilitated a further 1 log reduction. Cooking reduced *L. innocua* numbers on broccoli by an average of 1.1 log units and aerobic plate counts by between 1 and 2 log units. Microwave heating had a lethal effect on *L. innocua*. Combining chlorinated wash treatment, storage in polyvinyl chloride plastic covering and cooking induced a reduction in *L. innocua* and a combined treatment of washing with chlorine, storage in Modified Atmosphere Pacakging (5% $CO_2$, 5% $O_2$) for two days at 4°C and final microwave heating resulted in the lowest pathogen numbers, causing a 5.13 log cfu/g reduction. Whilst chlorine is effective in reducing *L. innocua* during minimal processing, it does not suffice alone to eliminate pathogens from vegetables, just as MAP storage is only effective as part of a hurdle procedure. Cooking is a valuable step for destroying *L. innocua* present on broccoli so as to ensure vegetables are safe for consumption.

*Keywords*: Minimal processing, washing, refrigerated storage, cooking, microwave, *L. innocua*, *L. monocytogenes*.

### *3.3.1. Introduction*

There is an increasing demand amongst consumers for fresh vegetables (Ramesh *et al*., 2002). Minimal processing limits the impact of processing on the nutritional and sensory qualities of fresh produce (Ohlsson & Bengtsson, 2002). It does, however, cause an increase in the risk of survival on the produce and subsequent infection of consumers by certain pathogens. Awareness is growing that fresh or minimally processed vegetables can be sources of pathogenic bacteria (Steele & Odumeru, 2004). *L. monocytogenes* is an organism that can survive in extreme environments, including at refrigeration temperatures (Critzer & Doyle 2010) and under the oxygen concentrations within modified atmosphere packaging (MAP) (Francis *et al*., 1999).

Broccoli sampled from fields has been found to contain *L. monocytogenes* (Gorski *et al*., 2003). Broccoli (*Brassica oleracea* var *botrytis)* is a member of the cabbage family. It has a rough surface and crevices in its structure (Frank, 2001) that retain water, so aiding in attachment of organisms to the crop. This protects organisms from the effect of sanitisers, making broccoli a possible substrate for organisms such as *L. monocytogenes* (Stine *et al*., 2005). Research on the specific behaviour of pathogens, their location in and on the plant surface, and means to treat products to eliminate these pathogens is limited (Brackett, 1999b).

The underlying principle of minimally processed ready-to-eat (RTE) products is the use of several hurdles at low intensities to attain a synergistic effect in controlling bacterial multiplication and spoilage on these RTE foods (Del Torre, *et al*., 2004). Such vegetables are washed and often dipped in a washing agent such as chlorine (Francis *et al*., 1999), before being packaged under conditions of modified atmosphere to increase the shelf-life and quality during refrigerated storage. It has been recommended that broccoli should be blanched in boiling water to maintain sensory quality during cooking (Ramesh *et al*., 2002). The ubiquity of *L. monocytogenes* in nature and its acknowledged presence in food-processing environments explain the difficulty in producing minimally processed foods free of the pathogen (Taormina & Beuchat, 2001).

As guidelines published by the South African Department of Health state that *L. monocytogenes* should be absent in one gram of raw vegetable produce

(Department of Health, 2006), eliminating any contamination from such produce that may be present after harvest is imperative during post-harvest processing.

The objective of this study was to determine the effect of minimal processing, which included washing with water and chlorinated water (200 ppm), and storage in modified atmosphere, followed by cooking in water or in-bag microwave heat treatment, on the survival of *L. innocua* on broccoli.

### *3.3.2. Materials and methods*

#### 3.3.2.1. Growth, isolation and maintenance of *L. innocua* culture

##### *3.3.2.1.1. L. innocua as surrogate organism for L. monocytogenes*

The processing phase was designed to follow the field trial phase of the study. During the field trial phase, *L. innocua* was used as surrogate organism to *L. monocytogenes* (O'Bryan *et al*., 2006), due to the practical constraints of working with a pathogenic organism in open conditions. The processing phase of the study was therefore continued with the use of *L. innocua* as the study organism.

##### *3.3.2.1.2. Bacterial inoculum preparation*

A *L. innocua* (serotype 6a, which has been fully sequenced (Nelson *et al*., 2004)) strain ATCC 33090 culture was obtained from Microbiologics (Minnesota, USA). The culture was streaked onto Tryptic Soy Agar and single colonies isolated after 48 h of incubation at 37°C.

A single colony was inoculated into 10 ml Tryptic Soy Broth (Biolab, Merck, South Africa), which was incubated in a shaking water bath (166 rpm) at 37°C for 18h to 20h, a time at which the culture was at a state of transition between the late logarithmic and early stationary phase of growth (Taormina & Beuchat, 2001). The bacterial cells were harvested by dispensing into 2 ml Eppendorf tubes, which were centrifuged for 10 min at 4000*g* (6600 rpm, RT150, Brake: 30) (Digicen20 centrifuge, Orto-Alresa). The supernatant was discarded and the harvested cell pellets were washed with three volumes of sterile saline solution (0.9% NaCl) before repeating

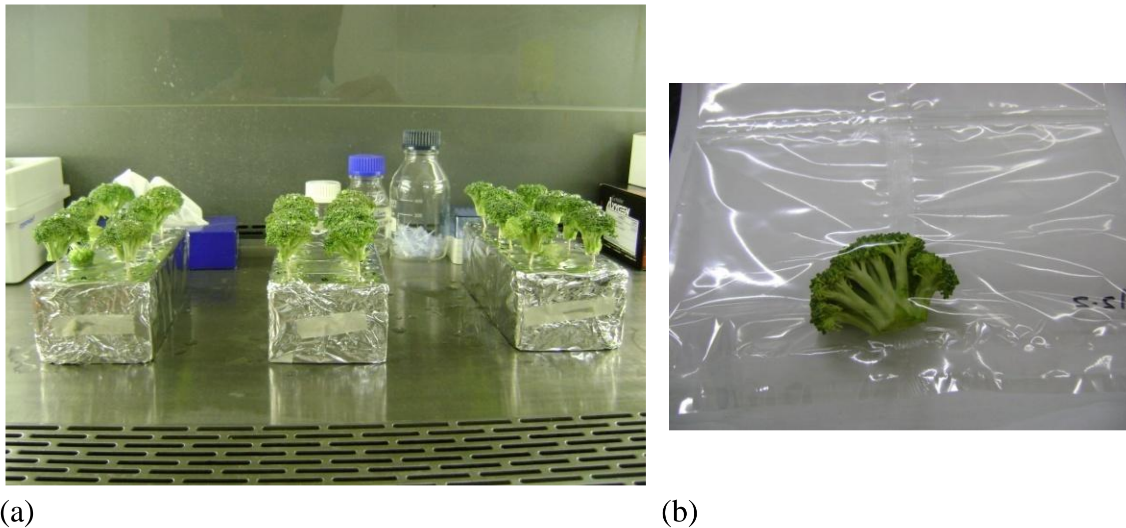
centrifugation. The supernatant was discarded again, the cells resuspended in saline solution at a cell density of $10^8$ cfu/ml, by means of comparison with the MacFarland standard (0.5) (Bhagwat, 2003) and from this a standard solution for inoculation prepared.

#### 3.3.2.2 Broccoli samples

The broccoli samples for the processing phase of the experimental study were obtained from a local green grocer in Pretoria, South Africa. The broccoli heads were enumerated for *L. innocua* and aerobic colony counts prior to processing treatments.

#### 3.3.2.3. Contamination of broccoli heads

Broccoli heads were cut into florets of 10 g weight and individually dipped into the sterile distilled water, inoculated at a cell density of $10^6$ cfu/ml *L. innocua*, for 30 s. In addition, 3 ml of the inoculated water was dripped onto the floret by means of a sterile syringe to ensure extended contact time of the organisms with the floret surface and to simulate contact conditions during actual processing. The florets were dried in a laminar flow cabinet for 30 min (Fig. 12).

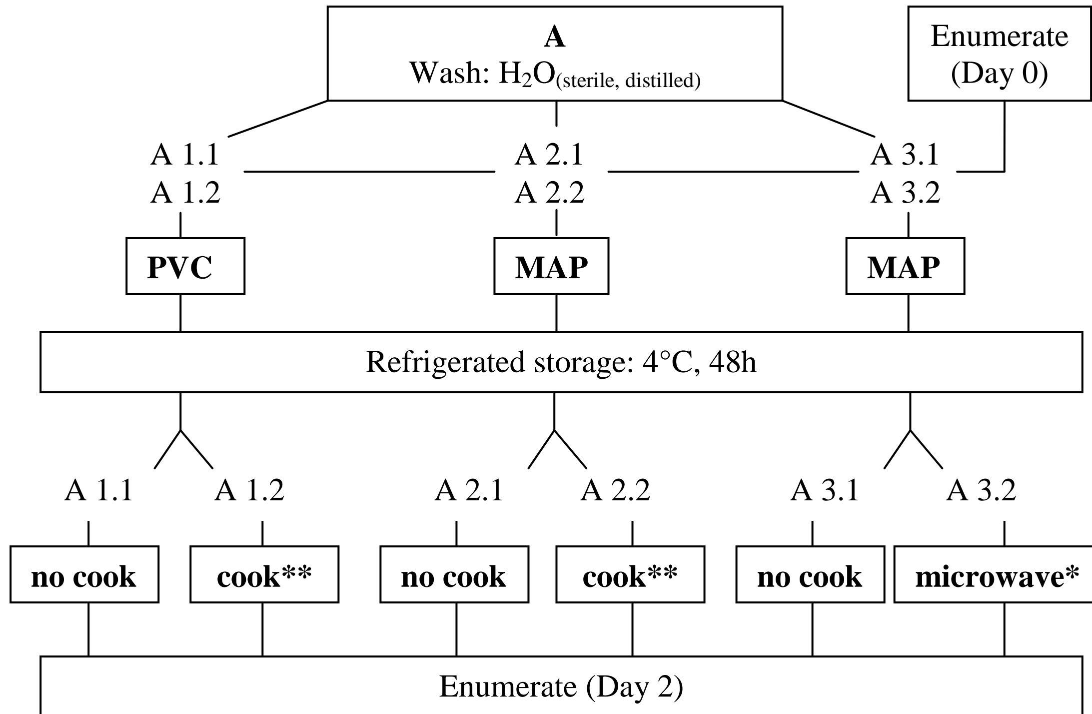


(a) (b)

**Figure 12** Minimal processing of the broccoli; a) experimental setup for contamination of the florets, mounted upright in laminar flow cabinet; b) single broccoli head in individual sealed package for MAP storage trial

### 3.3.2.4. Processing

Samples were subjected to different treatments to achieve certain final processing procedure combinations. Figure 13 provides a detailed illustration of the processing procedure for samples receiving a wash with sterile, distilled water (A) as first processing step. The same processing steps were followed for samples that were subjected to a chlorinated wash (B) as first processing step or samples that were not washed at all (C) in the first step. Each set of treatment combinations (A, B and C) contained 6 broccoli samples (A1.1, A1.2; A2.1; A2.2, A3.1, A3.2) that each received a different storage and heating sequence within the same wash treatment. Treatments 2 and 3 were stored under the same packaging conditions, but received different heat treatments, whilst treatment 1 was stored under different conditions but received the same heat treatment as Treatment 2. This experimental design facilitated the comparison of two different storage conditions as well as two different heat treatments.




PVC: Polyvinyl Chloride packaging

MAP: Modified Atmosphere Packaging

**cook**: 95±2°C, for 3 min

**microwave**: 850 W

**Figure 13** Processing treatment combinations of washing, packaging and cooking for Treatment combination A; B: Wash with chlorinated $H_2O$ (200 ppm); C: No Wash

3.3.2.4.1. *Minimal Processing*

*3.3.2.4.1.1. Washing*

The first step of the processing procedure involved a washing step, which employed any of three washing treatments of contaminated broccoli florets. In the first treatment (A), 8 florets contaminated with *L. innocua* were dried and washed. This was done by suspending each of the florets in 100 ml of sterile distilled water in a stomacher bag and gently shaking for 30 s. The florets were then dried in an upright position for 30 minutes in a laminar flow cabinet. For Treatment B, the florets were contaminated, dried and then washed with sterile, distilled water containing 200 ppm

chlorine. The chlorine solution was prepared from a 3.5% m/v sodium hypochlorite solution by adding 5.72 ml of Jik to 1000 ml of distilled water. After preparation, the solution was stored protected from light at 21 ± 2°C and used within 1 h of preparation. The same washing method was followed as for Treatment A. Treatment C served as control for the wash process and these florets were also contaminated and dried, but not rinsed after contamination.

*3.3.2.4.1.2. Packaging*

The florets packaged after washing, under two different packaging conditions. Two florets from each wash treatment were packaged on polystyrene plates and covered with PVC cling wrap. The remaining florets were packaged under modified atmosphere conditions in polypropylene bags of 20 cm x 20 cm size, suitable for vegetable modified atmosphere packaging, obtained from Packaging World, Pinetown, South Africa. The film was non-perforated, with the characteristics as shown in Table 8.

**Table 8** Characteristics of commercial MAP packaging film used in the study

| **Thickness (µm)** | **Permeability $O_2$ ($ml.m^{-2}.d^{-1}.atm^{-1}$)** | **Permeability $CO_2$ ($ml.m^{-2}.d^{-1}.atm^{-1}$)** |
|---|---|---|
| 20 | 1600 | 3600 |

(Serrano *et al.*, 2006)

The modified atmosphere was obtained by filling the packages with a gas mixture containing 5% $CO_2$, 5% $O_2$ and 90% $NO_2$ and sealing the packages with heat (Multivac A300 vacuum sealer). One package from each of the three wash treatments was kept, unrefrigerated, for 30 min after sealing and the concentration of oxygen and carbon dioxide inside the packages monitored using a Gaspace 2 gas analyser (Systech Instruments Ltd., Thame, Oxfordshire, UK). A syringe was inserted into the package through a rubber seal placed on the film. The instrument was calibrated towards air. Packages were then stored under refrigeration at 4°C for 48 hours.

3.3.2.4.2. *Cooking treatment*

After completion of the 48h refrigerated storage, the florets were subjected to different cooking treatments. Each treatment had an uncooked control (1.1, 2.1 and 3.1, Fig. 13). Florets were submitted to cooking with boiling water (1.2 and 2.2, Fig. 13) or florets were heated in the microwave (3.2, Fig. 13).

Conventional home cooking of broccoli is recommended by commercial food producers, to be performed by placing the broccoli florets in lightly salted water and heating in boiling water for 10 min. The method followed in this study saw the broccoli florets submerged in boiling water for a period of 3 min, contained in their 20 cm x 20 cm plastic packages. The packages were kept sealed for the duration of the cooking procedure. The cooking time was determined in preliminary studies as a time that produces broccoli florets of a desired sensory quality in terms of green colour of the floret as well as softness of the floret, criteria that would be used in domestic cooking circumstances in addition to the cooking instructions recommended by the manufacturer. The florets were kept in the sealed packages so as to eliminate a further additional wash effect during the cooking procedure and therefore to prevent an additional variable in the experimental design.

The water cooking procedure was carried out by placing the florets in their sealed packaging into a boiling water bath at 100°C, facilitating a steam blanching effect at 95±2°C, for 3 min. Thereafter they were enumerated for the population of *L. innocua*.

Cooking instructions for broccoli found on commercially sold packages for in-bag microwave cooking, recommend a cooking time of 3 min in an 850 – 950 W microwave oven. This serves to cook the broccoli to a level of desired sensory quality. In preliminary studies in the present work the commercially recommended cooking time was found to be too long, as it resulted in severe browning of the florets at the stem and necessitated shortening of the microwave cooking time to 30s. The observed browning of the broccoli stems could be ascribed to the sample size submitted to the heating process. The florets, at a weight of approximately 10g each, were packaged individually in packets of 20 cm x 20 cm after contamination and minimal processing before refrigerated storage. They were then heated individually in these packages.

For the microwave cooking procedure the packages were cut open on the upper left corner by cutting off a 3cm by 3cm triangle. The packages were then placed in the centre of a domestic microwave oven at 2450 MHz with a maximum output power of 850 W and exposed to microwaves at full power for 30 s before they were removed and processed for enumeration. The microwave cooking was performed so as to simulate an "in-bag" microwave cooking effect of samples stored in the cooking bags prior, therefore the PVC samples were only cooked conventionally and not by means of microwave heating as well.

### 3.3.2.5. Enumeration

The population of *L. innocua* on the broccoli florets were enumerated after each treatment to assess its effectiveness in sanitizing the vegatetable.

Broccoli samples of 10 g weight were stomached for 2 min in 90 ml Buffered Peptone Water (0.1%) (Merck), from which serial dilutions were prepared and spread plated onto *Listeria* Selective media (Oxford formulation) (Oxoid) for *L. innocua* enumeration. APC analysis was done by pour-plating with Plate Count Agar (PCA) and a cover-layer of Bacteriological Agar (Biolab, Merck). Each of the samples were analysed with triplicate plate counts from the serial dilution. The plates were incubated at 37°C for 24 to 48h to allow enumeration of *L. innocua* colonies. Typical colonies were confirmed on Chromogenic *Listeria* Agar Base medium (Oxoid).

### 3.3.2.7. Statistical analysis

Three replicate experiments were conducted for each trial. All the samples used for colony enumeration, including controls were prepared in duplicate. The mean values from three experimental repetitions, with triplicate plate counts of duplicate samples were calculated and reported with 99% confidence interval. Data were subjected to analysis of variance (ANOVA) (Han, *et al.*, 2000) and mean comparisons were performed to examine if differences of variables were significant ($P< 0.05$), so as to assess the effect on the survival of *L. innocua* on the broccoli surface and therefore

the efficacy of the individual treatments on the reduction of food safety risk during the minimal processing procedure.

Treatment variances were not homogeneous; therefore significance testing was done at the 1% level (M. Smith, personal communication, 2009).

### *3.3.3. Results*

#### 3.3.3.1. Wash treatment: Individual effect of washing with water and chlorinated water

Washing with water, as determined directly after wash treatment on Day 0 and comparing numbers from treated samples with control values, reduced the *L. innocua* numbers on broccoli by 1 log unit to 4.23 log cfu/g. The chlorine treatment reduced the numbers by 2 log units to 3.11 log cfu/g, as determined 30 minutes after exposure of the contaminated broccoli to the treatment (Fig. 14). The differences between the *L. innocua* numbers before and after treatment were, however, not statistically significant ($P > 0.01$).

The APC were not significantly affected by the wash treatments (Fig. 14).

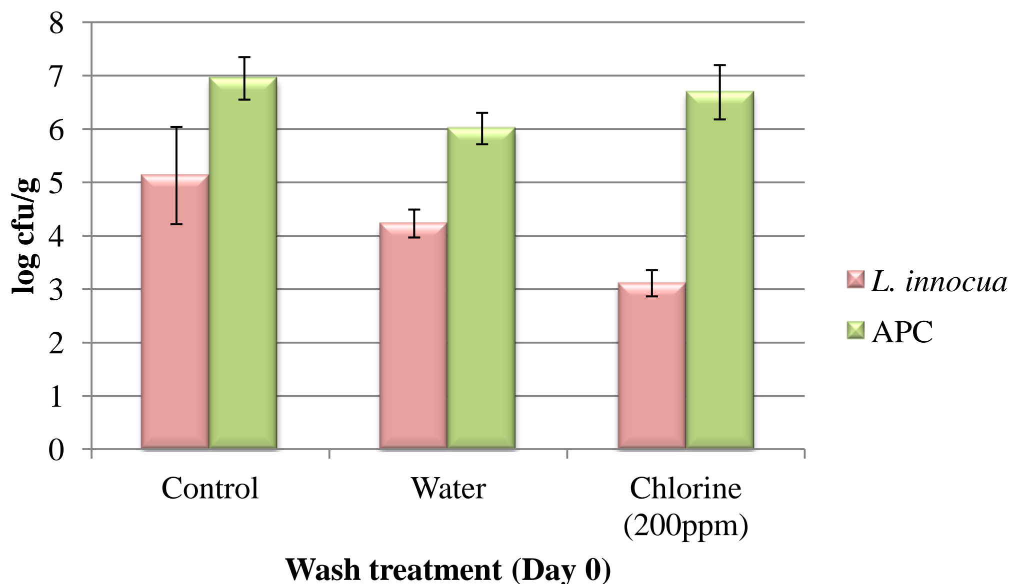



**Figure 14** Individual effect of washing treatments on microbial counts on broccoli on Day 0 (D0) (± standard deviation), n=3

#### 3.3.3.2. Wash treatment: Main effect of washing as part of treatment combination

The main effect of washing (as measured on Day 2) includes the effect of storage and cooking on the washed samples and thus evaluates the influence of the washing as part of the processing procedure (as opposed to the individual effect of washing as a single treatment, as measured on Day 0). These effects are represented by Fig. 15. Washing with water facilitated a 1 log reduction, to a level of 2.37 log cfu/g, in *L. innocua* numbers compared to the unwashed sample (control) (Fig. 15). Washing with 200 ppm chlorine induced 2 log reduction, to a level of 1.46 log cfu/g, in cell numbers when compared to the unwashed control sample. The differences between the *L. innocua* numbers as well as between APC after the treatments were, however, not statistically significant ($P > 0.01$). When only the trend in cell number changes were studied, it was deduced that the broccoli sample washed with water showed a slight increase in microbial load in comparison to the unwashed sample, whereas the total colony count on broccoli washed with chlorine was somewhat lower (less than 1 log unit), (Fig. 15).

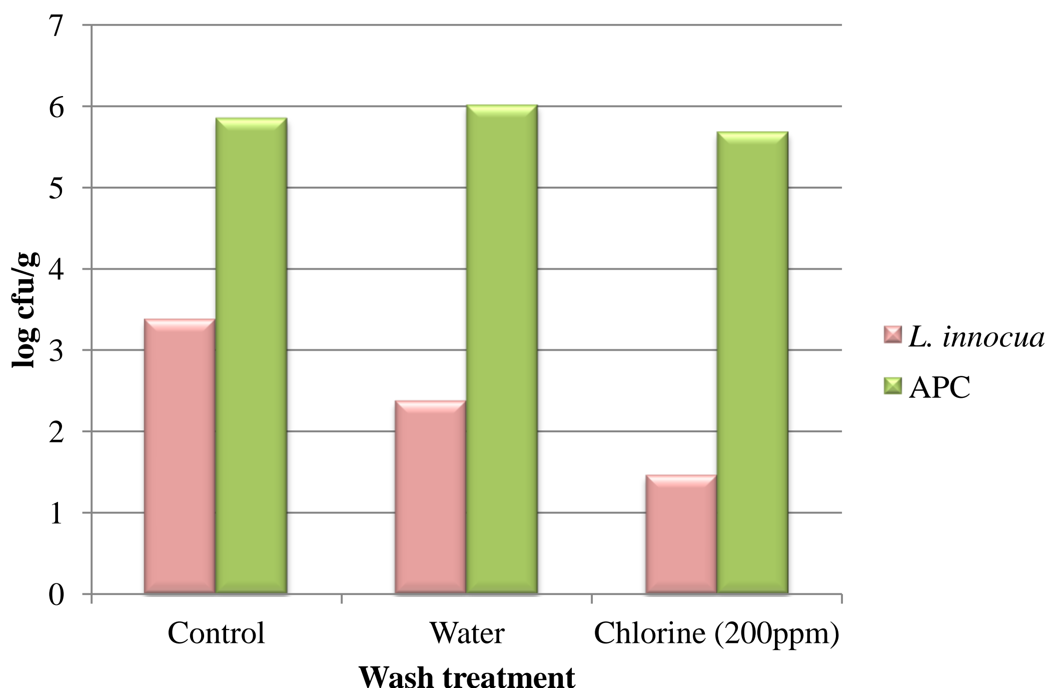



**Figure 15** The effect of washing with water and chlorine (results on Day 2), compared to a control on *L. innocua* (standard error of means: ±0.546) and APC (standard error of means: ±0.499) on broccoli

#### 3.3.3.3. Cooking treatment

Microwave cooking (treatment combination 3.2, Fig. 16), had a lethal effect on *L. innocua* and the numbers after this treatment were not only significantly lower than the uncooked sample (3.1, Fig. 16), but also significantly lower than the resulting *L. innocua* numbers from cooking treatment ($P < 0.001$) (2.2, Fig. 16). APC numbers after microwave treatment were also lower than on the uncooked sample (3.1, Fig. 16) and lower than the numbers after cooking treatment (2.2, Fig. 16). Microwaving samples (in-bag) for 30 s resulted in total destruction of *L. innocua* on broccoli (3.2, Fig. 16), as no viable organisms were detected on the sample after treatment, causing a reduction of 3.37 log units.

Cooking treatment (1.2 and 2.2, F. 16), resulted in *L. innocua* counts that were lower in numerical value than those that were not cooked (1.1 and 2.1, Fig. 16), even though these differences were not statistically significant ($P > 0.01$). Cooking reduced *L. innocua* numbers on broccoli by an average of 1.1 log units and APC by between 1 and 2 log units (Fig. 16).

Treatments 2.1 and 3.1 (Fig. 16), were identical and served as uncooked controls for 2.2 and 3.2. Differences between *L. innocua* and APC counts for 2.1 and 3.1 were similar. Exact cell numbers differed due to the intrinsic variation in initial inoculation level which is to be expected when working with live organisms and samples are contaminated individually.

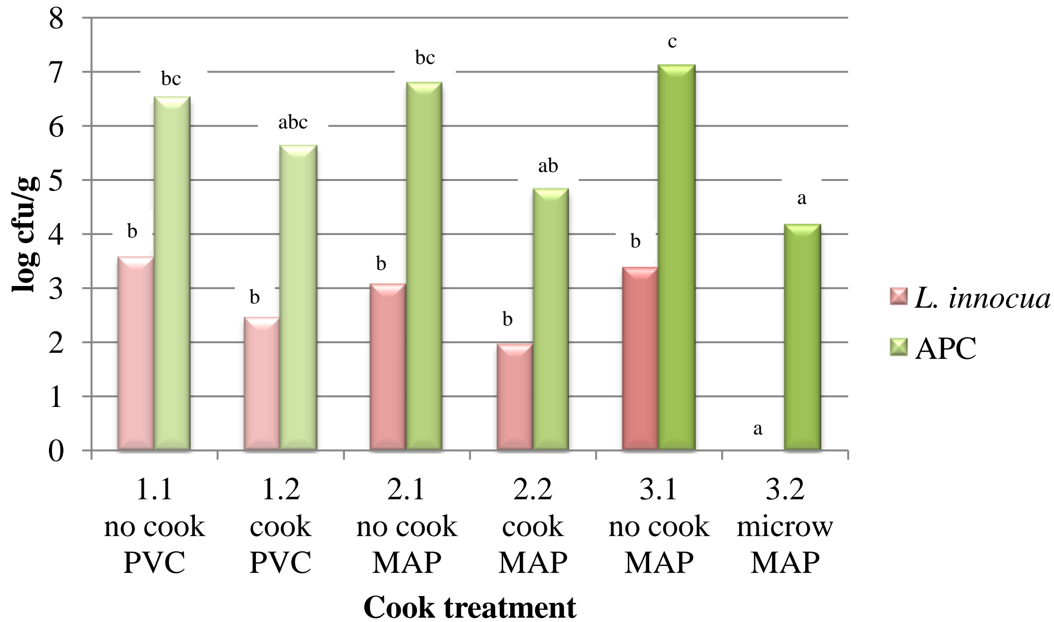



**Figure 16** The effect of cooking on mean *L. innocua* numbers (standard error of means: ±0.452) and APC (standard error of means: ± 0.549) on broccoli; Same coloured columns with the same letter are not significantly different from each other ($P$> 0.01)

### 3.3.3.4. Total effect of minimal processing and cooking on *L. innocua*

Refer to Fig. 13 for the processing procedure experimental design to provide clarity on the following data. Comparing the same storage and cooking conditions, but different wash treatments, provided insight into the main effect of washing and how this effect is influenced by changing the other processing parameters (compare same coloured columns for A, B and C, Fig. 17). For samples stored under atmospheric conditions (PVC), not cooked after storage (1.1, Fig. 17), *L. innocua* numbers after chlorine wash (log 2.13 cfu/g) were less than after water wash (log 2.71 cfu/g), and also substantially less than the numbers on the unwashed sample (log 4.86 cfu/g). Cooking samples after atmospheric storage (1.2, Fig. 17) also resulted in cell numbers for samples washed with chlorine (1.66 log cfu/g) to be lower than those washed with water (2.31 log cfu/g) and substantially lower than the unwashed sample (2.37 log cfu/g). Samples stored under MAP conditions after wash treatment without final cooking (2.1, Fig. 17), followed the same trend for the wash effects, with the cell numbers on the sample washed with chlorinated water (1.74 log cfu/g) being lower

than on the water washed sample (2.34 log cfu/g) and both of these were substantially less than the numbers on the unwashed sample (5.11 log cfu/g). Samples that were stored under MAP and then cooked (2.2, Fig. 17), displayed cell numbers for chlorine treatment (1.63 log cfu/g) that were not less than on water washed samples (1.72 log cfu/g). Submitting the samples to microwave treatment (3.2, Fig. 17) had a lethal effect on *L. innocua* cells and this final treatment step incurred a significant reduction compared to the unmicrowaved samples (3.1).

After water wash treatment (A) the *L. innocua* numbers on the uncooked sample stored under MAP (2.1, Fig. 17) displayed a high level of variability, making the difference from the PVC sample (1.1, Fig. 17) insignificant, (also due to the large standard deviations within the treatments). MAP combined with cooking (log 1.32 cfu/g) (2.2, Fig. 17) resulted in less cells than a combination of PVC and cooking (log 2.31 cfu/g) (1.2, Fig. 17).

The results after the chlorine wash treatment (B) indicated that the different storage and cooking conditions within this wash treatment did not differ significantly, with only the numbers on uncooked chlorinated samples packaged in PVC (log 2.13 cfu/g) (1.1, Fig. 17) being slightly higher than those receiving other additional treatments. The chlorine treated, PVC stored, cooked samples (1.2, Fig. 17), had lower *L. innocua* values (numerically) (log 1.66 cfu/g) than those not washed (C 1.2) (log 3.37 cfu/g) or only washed with water (A 1.2) (log 2.31 cfu/g) before cooking.

Numbers on the unwashed (C), uncooked samples were not significantly different from each other (log 4.86 cfu/g (1.1), log 5.11 cfu/g (2.1) and log 4.33 cfu/g (3.1); $P > 0.01$). The samples that received cook treatments were different from those that did not receive cooking treatment after both storage conditions (log 4.86 cfu/g vs log 3.37 cfu/g for PVC storage (1.1 vs 1.2); log 5.11 cfu/g vs log 2.52 cfu/gfor MAP storage (2.1 vs 2.2) and log 4.33 cfu/g vs 0 log cfu/g for MAP with microwave cooking (3.1 vs 3.2)). Combining MAP storage and cooking afterwards (log 2.52 cfu/g) (2.2), did result in lower numbers than the combination of PVC storage and subsequent cooking (log 3.37 cfu/g) (1.2).

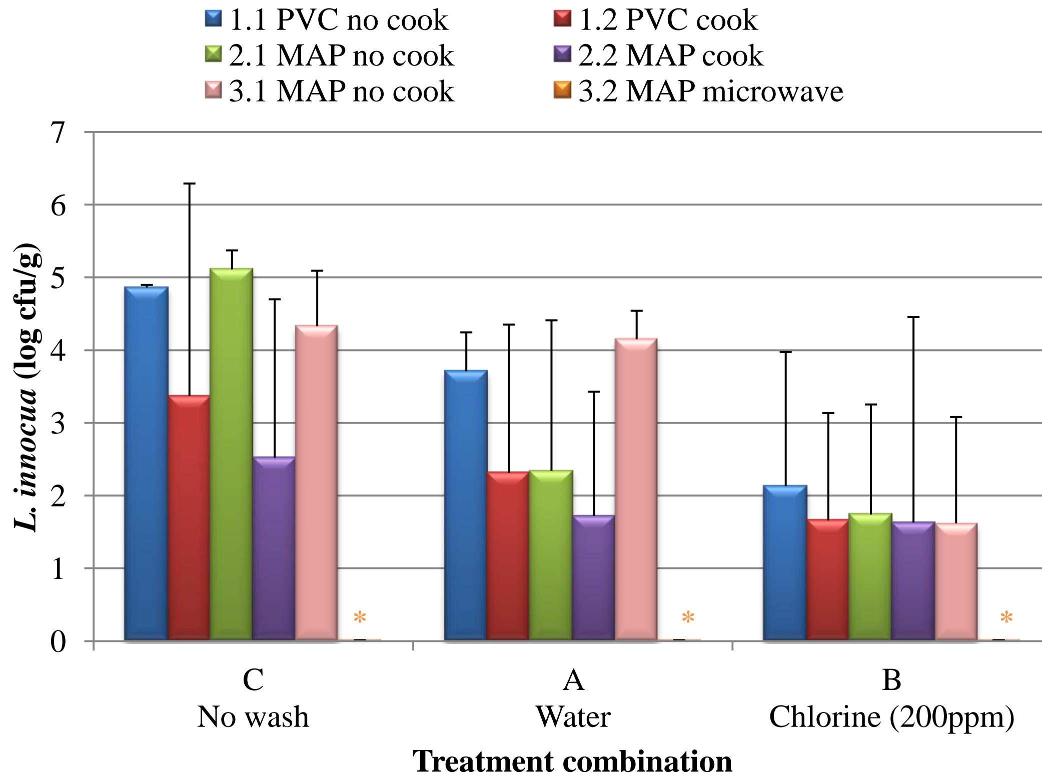



**Figure 17** The effect of minimal processing, (no washing (C) washing with water (A), and chlorine (B), followed by refrigerated storage) and cooking on *L. innocua* on broccoli; *Count below limit of detection.

### 3.3.3.5. Total effect of minimal processing and cooking on aerobic plate count

APC numbers on uncooked samples were similar for unwashed (C) and for washed (A) treatments ($P > 0.01$), as displayed in Fig. 18. Microbial counts on broccoli were reduced by cooking without wash treatment as well as after water and chlorine wash treatments and also after being packaged in PVC and stored under modified atmosphere. Cell numbers decreased substantially after combined treatments of chlorine wash, MAP storage and cooking (B 2.2, Fig.18), as well as after chlorine wash, MAP storage and microwave cooking (B 3.2, Fig. 18).

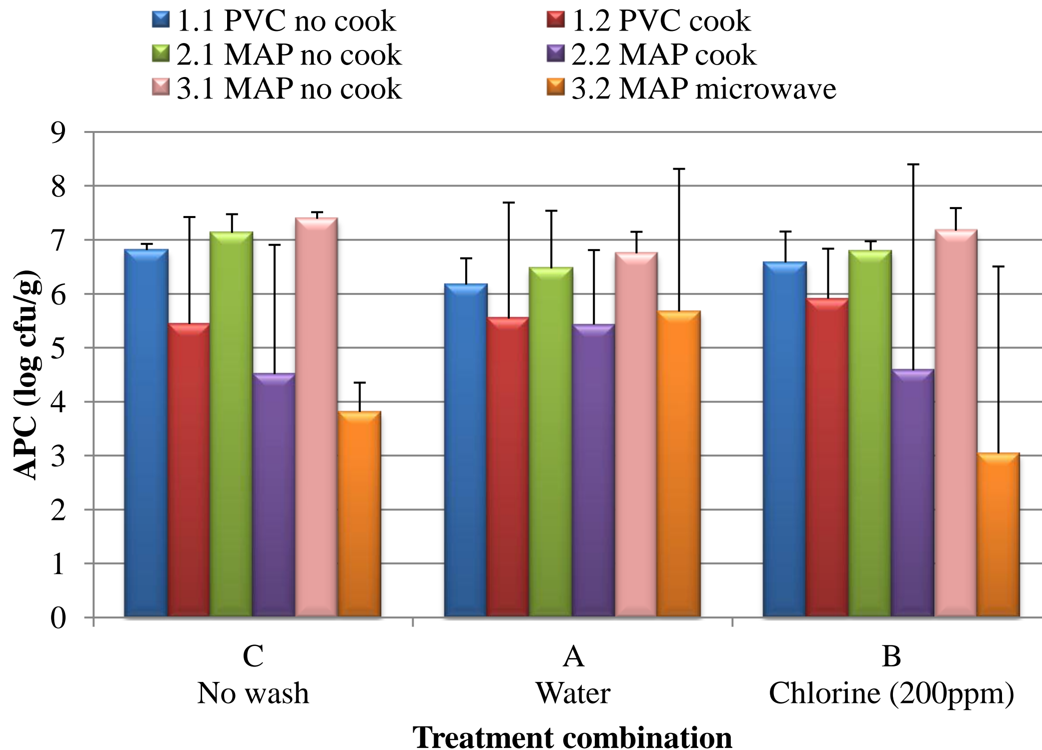



**Figure 18** The effect of minimal processing, (washing with water (A), chlorine (B) or no washing (C), followed by refrigerated storage) and cooking on APC on broccoli

### *3.3.4. Discussion*

Chlorinated water washing had a greater effect on *L. innocua* numbers than washing with water. This is in agreement with results from various studies, including those of Brackett (1987), Francis *et al*. (1999) and Zhang & Farber (1996), who reported that counts of *L. monocytogenes* were reduced in the order of 10 times higher by chlorine than those washed with water. Ells & Hansen (2006) and Gorski *et al*. (2003), reported that washing with water does remove unattached organisms, whilst chlorine further kills or injures cells (Day 0).

The difference between the APC counts on the on the broccoli after wash treatment (Day 0) were insignificant ($P > 0.01$) and in addition to that, not corresponding with the trend observed for the *L. innocua* on the broccoli. This suggested that the autochthonous flora was not affected by the wash treatments. Soriano *et al*. (2000) and Ukuku & Fett (2002), also found that wash treatment with distilled water incurred no significant decreases in aerobic microorganisms. Schoeller *et al*. (2002), suggested vegetable organic matter could have decreased the active

chlorine in wash solutions, causing the chlorine rinse employed to be less effective. Other influential factors could be the presence of hydrophobic pockets on the broccoli surface or crevices in the broccoli surface structure that create an isolated habitat for the microorganisms and allow them to escape the effect of the chlorine (Adams *et al*., 1989). The reduced effect of chlorine could also be attributed to resistance of the bacteria to the chemicals (Lisle *et al*., 1998).

For practical research purposes the initial *L. innocua* level inoculated onto the broccoli was high. Final cell counts may therefore not reflect microbial levels actually encountered in the field (Flessa *et al*. 2005). This had to be kept in mind when comparing bacterial levels on the produce with food safety standards such as the Guidelines published by the South African Department of Health.

Cooking in boiling water resulted (Day 2) in a reduction in the numbers of *L. innocua* on broccoli and the aerobic bacterial counts, which was in agreement with the results of Mazzotta (2001), who established that submitting broccoli florets to heat at 75°C for 10s had an antilisterial effect (Lund *et al*., 1998).

Microwave treatment (30s) (Day 2) had the greatest effect on *L. innocua* numbers on broccoli. Rodriguez-Marval *et al*. (2009), also reported that heat treatment by means of microwaving at high power for 75s inactivated *L. monocytogenes*, with Woo *et al*. (2000) attributing the effect to membrane damage in gram positive organisms. The reduction in APC induced by microwave heating indicates that microwaving does have a greater destructive effect on the microbial population on the vegetable than conventional cooking.

Even though analysis of variance (Day 2) revealed the effect of wash-cook treatment combinations on the *L. innocua* not to be significantly different from each other statistically, the numerical differences did still suggest influences of the treatments and treatment combinations on the *L. innocua* survival on the broccoli post-harvest. Each individual combination of treatments could be analysed and compared to a different combination separately so as to assess the effect and efficacy thereof in the management of pathogen contamination of vegetables.

Chlorine treatment (200 ppm) had the greatest diminishing effect on *L. innocua* counts, whilst washing with water also seemed to facilitate a lowering of *L. innocua* numbers in comparison to the unwashed samples. Ukuku & Fett (2002), found that washing with water did not cause a significant reduction of *L. monocytogenes* on fresh produce surface, but chlorine treatment did.

Results from the water and chlorine washed samples analysed on Day 2, cooked after atmospheric storage, indicate that the chlorine treatment could have sensitised cells to heating, as was suggested by Taormina & Beuchat (2001), but it is also possible that after chlorine treatment, less cells were present than on the water washed sample due to the effect of chlorine in addition to the rinsing effect of the water and so subsequent cooking resulted in lower final numbers on the chlorinated sample.

MAP storage without other minimal processing steps (such as water or chlorinated washing) did not have an effect different from that of storage under atmospheric conditions, but in combination with water or chlorine wash, the numbers for MAP are lower than for PVC (Zeitoun & Debevere, 1991). Cooking did not display a synergist effect with MAP, as the numbers were similar to the PVC, cooked samples and to the water washed, cooked samples. This agrees with the findings of Kakiomenou *et al.* (1998), with regards to the hurdle effect of MAP. *L. innocua* numbers on the broccoli did not increase during the 2 days refrigerated storage. Schoeller *et al.* (2002) and Flessa *et al.* (2005), made the same observation even after 7 days refrigerated storage. Duh & Schaffner (1993), did however, conclude that refrigerated storage alone cannot ensure that the growth of *L. monocytogenes* will not occur.

The effect of the water wash treatment reflects the behaviour of the attached organisms, as washing should have removed any unattached cells. In comparing the water washed samples with each other, the analysis of storage conditions in this wash treatment was inconclusive. Variations in values were large for MAP stored, uncooked samples and gave large standard deviations within treatments. The MAP, cooked samples were one log unit lower than the PVC cooked sample, but this could rather be ascribed to the effect of cooking, than to synergism between MAP and heat treatment.

The similarity of the final cell numbers of samples exposed to chlorinated wash treatment, suggest that the effect of chlorine is the main effect in the hurdle system. Chlorine alone does have an effect, but produces a better result in combination with other hurdles. This was seen by the fact that if the chlorine treatment was followed by storage in PVC and the sample was not cooked, the numbers were slightly higher than in the case where MAP storage followed chlorination and samples were cooked. Zeitoun & Debevere (1991), found that *L.*

*monocytogenes* numbers increased during MAP storage (90% $CO_2$, 10% $O_2$), but that the MAP did have a synergistic effect with a decontaminant. This $CO_2$ concentration is, however, much higher than MAP conditions for broccoli storage applied in this study, which would therefore cause a different bacteriostatic effect, explaining why this prominent synergism was not observed. Chlorine treated, cooked samples had lower values than those not chlorinated, which indicates a possible sensitisation of cells by chlorine to heat. Taormina & Beuchat (2001) proposed a theory that two subpopulations of *L. monocytogenes* cells are created as a result of chlorine stress. This involves the weaker, cells dying, as they are less resistant, with only the more stable cells which are perhaps approaching stationary phase, remaining and succeeding in surviving thermal treatment. Within the chlorine treatment however the cooked values did not differ significantly from uncooked values, which might be because the cells surviving chlorination are more resistant, rather than being more sensitive or that the effect of chlorine was already so pronounced that the cooking effect was not significant in terms of the numbers of cells that remained after washing. Sublethal injury by one treatment could also induce a stress response in the pathogen, making it more resistant to a subsequent treatment. Bunduki *et al*. (1995), observed that chlorine seemed to cause such a degree of injury, as an extended time was needed for cell repair.

The unwashed, uncooked samples showed the effect of the refrigerated storage only. Storing the broccoli under MAP conditions does not have a different effect on *L. innocua* growth or survival than storage under atmospheric conditions in PVC. Kakiomenou *et al*. (1998), also indicated that packaging in modified atmospheres is not necessarily of greater value as an additional hurdle for growth of *L. monocytogenes* in comparison to conventional packaging under aerobic conditions. As *Listeria* is a facultative anaerobe, conditions of modified atmosphere may not influence it markedly.

The main effect of heat treatment on microbial safety during broccoli preparation was evident from the reduced numbers after cooking. Microwave treatment of the *L. innocua* on broccoli proved to be lethal, possibly due to the damaging effect of microwave radiation on the membrane of the organism (Woo *et al*., 2000).

The effect of cooking on the aerobic microbial load is evident. Samples from all wash treatments and packaging conditions exhibited lower numbers after receiving

heat treatment. As the effect of cooking is however, small, this could suggest the presence of spores within the microbial population, as these are more resistant to the effect of heat (Haas *et al*., 1995).

Samples exposed to chlorine and stored under MAP had slightly lower numbers than those that received a water wash treatment, but not substantially so compared to the unwashed samples. This suggests that the effect of chlorine on the APC was not the main influencing factor in the diminishing of cells, which is supported by the findings of Soriano *et al*. (2000).

From the fact that the uncooked samples within the different wash treatments did not differ significantly from each other for APC, it is deduced that the autochthonous flora is less affected by chlorine, than is the case for *L. innocua*. This suggests that the background flora contains organisms that might better attach to the surface and some present may also survive well under modified atmosphere. The possibility exists that the organisms might have a good recovery mechanism, but a sensitising effect to heat by chlorine could still be a factor, especially in combination with MAP. Nguyen-the & Carlin (1994), also stated that the composition of mesophilic microflora was not significantly altered in end products, indicating that processing did not greatly affect the population.

The combination of chlorine washing, MAP storage and microwave cooking seemed to be the best for reduction of total microbial load on broccoli, although even in the absence of a wash treatment, reduction was also achieved.

### *3.3.5. Conclusions*

Contamination of vegetables with pathogenic organisms can occur pre-harvest and at numerous places during post-harvest handling before and after purchase by the consumer. Minimal processing procedures were seen to have an effect on the level of *L. innocua* present on broccoli post-harvest.

Washing broccoli with chlorinated water (200 ppm) may reduce *L. innocua* numbers on broccoli more so than washing with unchlorinated water, but not eliminate the pathogen from the crop surface. Cooking broccoli succeeds in reducing *L. innocua* numbers on broccoli, whilst microwave heating is lethal to the pathogen. In this study, combined treatments of washing with chlorine, storage under conditions

of modified atmosphere (MAP: 5% $CO_2$, 5% $O_2$) and reduced temperature (4°C) post-harvest and before consumption, with final microwave heating results in the lowest pathogen numbers.

Although individual processing steps can reduce *L. innocua* numbers on broccoli, intermediary cross-contamination can occur even after a minimal processing step such as washing. Survival was also seen to be possible under home storage conditions. With *L. innocua* as surrogate organism to *L. monocytogenes*, it can be deduced that minimal processing procedures can be more effective in preventing the survival of pathogens such as *L. monocytogenes* on broccoli up to the point of sale and consumption when implemented in combination and as part of a hurdle system.

# CHAPTER 4 GENERAL DISCUSSION

The main objective of this study was to determine the survival of *L. innocua,* as surrogate organism for the pathogen *L. monocytogenes,* on broccoli, after application by means of spray irrigation with contaminated water, firstly in field conditions pre-harvest and subsequently during post-harvest processing. *L. monocytogenes* is a ubiquitous pathogen that is able to survive under adverse conditions (Pearson & Marth, 1990). For this reason, its survival was assessed, by means of *L. innocua* as surrogate organism, under field conditions, with monitoring of environmental parameters. Post-harvest, broccoli undergoes minimal processing (Hill *et al*., 2002), necessitating the investigation of the effects of chlorinated washing, modified atmosphere packaged storage and subsequent cooking on the survival of *L. monocytogenes* on the vegetable.

Broccoli is a vegetable substrate that supports the growth of pathogenic microorganisms, as its surface structure has crevices in which microorganisms present on the crop surface are protected from the effect of sanitisers. This increases the likelihood that the organisms may survive on the surface of the crop and subsequently transmit disease to the consumer (Chmielewski & Frank, 2003). The latest trend in the food industry, amongst consumers and therefore amongst producers, is towards health. Demand has therefore increased for minimally processed foods grown without pesticides and not preserved by any chemical substances or prepared so as to retain their nutritional content.

Vegetables are sold in their natural, unprocessed form and are even consumed raw to retain optimum nutritional quality. This increases the risk of pathogens present on the crops in the field being transferred to the consumer upon consumption. Vegetable crops that are traditionally cooked before consumption, such as broccoli, have therefore become crops carrying increased risk as sources of pathogenic organisms. Due to this change in dietary habits and the subsequent unexpected associated increased risk, broccoli was chosen as the vegetable substrate for this study. Broccoli is a crop susceptible to white rot, making it difficult to grow under organic conditions. This proved to be problematic, as various heads were affected by rot even in the period before inoculation treatments with the pathogen commenced. These crops were excluded from the experimental crops and did therefore not affect

data. Experiments could have been better performed had crops been grown by an experienced farmer under established vegetable growth conditions. This could have ensured broccoli crops of more suitable size and shape for use as experimental samples resulting in greater consistency between repetitions and closer resemblance to actual commercial conditions. Less crop loss and thus of loss of experimental samples would have been experienced.

Broccoli has a hydrophobic surface, which complicates contact of the inoculated fluid with the crop. Great amounts of water flow off the sample upon application, which made it difficult to establish how much pathogen was finally applied to the crop. This also resulted in great variances in initial cell numbers between crops within treatments and between different repetitions. This obscures results of final numbers and causes variation and confusion when conclusions have to be made by comparing final cell counts in different treatments. Bacterial behaviour has also been shown to vary under similar conditions, depending on the initial bacterial load.

Broccoli is a vegetable crop grown and harvested in the winter months (Cliff *et al*., 1997). During this time, pathogens present on the crop are exposed to adverse environmental conditions, especially in the Highveld area of South Africa, where low temperatures are reached during the night with the possibility of frost, and dry conditions prevail (Anon., 2011c). The non-fastidious nature of *L. monocytogenes* renders it a pathogen with the ability to proliferate in extreme environments. *Listeria* species are therefore well suited for growth and survival under various conditions of exposure on food product surfaces and food processing facilities (Chmielewski & Frank, 2003). The presence of the pathogen in soil and on raw products introduces *Listeri*a into the vegetable processing facilities. Outbreaks of listeriosis have been associated with broccoli and coleslaw, demonstrating the potential hazard and accompanying risk that presence of the pathogen on and around fresh produce poses (Hines 1999, Kuntz 1995). *Listeria monocytogenes* was therefore selected as the target organism for the survival study.

The use of the surrogate organism *L. innocua* was rendered necessary by the risk associated with using a pathogen in open field trial conditions. Even though conclusions are made and assumed to be applicable to the pathogenic organism, the possibility does exist that differences in behaviour between the organisms could occur. For studies of specific bacterial behaviour, such as growth kinetics under

certain imposed environmental conditions, use of the pathogen for the studies would be more suitable, to ensure correct conclusions to be made, especially in terms of food safety.

In field trial conditions, many external variables exist which are uncontrollable. The trial is carried out in open atmosphere and the experimental specimens are therefore exposed to and influenced by external factors of various kinds (Girardin *et al*., 2005). The simultaneous variation of, in the case of this study, the environmental parameters of temperature and humidity, complicate the drawing of a conclusion on the effect of individual parameters on the survival of the organism. Controlling these parameters to remain constant over three repetitions is also impossible, as these parameters fluctuate naturally at different times. This caused the only constant variable to be the inoculation level, explaining why the most significant correlations were perceived between bacterial counts and time. The resulting variability over the replicates of the bacterial counts on the broccoli during growth of the crop in the field influenced the experimental results with respect to the standard error margin.

The trials would have benefitted from a greater degree of control over the experimental field plot conditions to establish the influence of environmental parameters on growth kinetics of the pathogen present on the crop. In order to achieve this controlled scenario, trials would have had to be performed in confined spaces, ruling out the possibility of study under actual field conditions. Simultaneous trials in a greenhouse environment could have provided great insight into the efficacy of treatments in the field in comparison to a condition-controlled environment, as well as the difference in microbial behaviour in the field and the laboratory. Performing experiments in such controlled environments would have enabled the study of the effect of individual environmental parameters. Examples of these are the effect of elevated or cold temperatures, high or low moisture conditions as well as the effect of UV exposure on the pathogen, alone and in combination. This would enable the effective deduction of the result on growth kinetics of the exposed organism and comparison to field conditions. More frequent sampling, despite requiring a greater amount of sampling and the constraints imposed by preparation and analysis time, would have resulted in more condensed growth curves providing information on daily fluctuations in cell numbers and correlation with changes in daily and nightly temperatures.

Other external factors included birds, which threatened the broccoli crops, necessitating the erection of netting over the field plots. The possibility exists that the netting could have influenced the exposure of the crops to the natural UV radiation and thus the conclusion of the influence of maximum daily temperatures on the organism survival on the crop. Spoilage by insects was another problematic influencing factor. The produce was to be produced under organic conditions as far as possible, simply to exclude the effect of pesticides on the pathogen under investigation.

The microscopy study did not provide much insight into the bacterial attachment, but the smooth, waxy surface of the broccoli could be visualised. No insight was gained into the attachment of the *L. innocua* cells to the broccoli surface or into the effect of washing and chlorinated washing on the attached state of the organism.

As it has been proven in other studies that *L. monocytogenes* does attach to broccoli surface and this has been visualised under a scanning electron microscope (Kalmokoff *et al*., 2001), the infrequent detection of the organism on the vegetable surface could be attributed to less than optimal attachment conditions during the experimental procedure, such as sub-optimal temperature conditions (Gorski *et al*., 2003) or insufficient time to form biofilms as method of attachment (Critzer & Doyle 2010). The hydrophobic surface of the broccoli could also have been responsible for the prevention of attachment during the contact time, as it has been proven by Ren *et al*., (2007) that broccoli has a waxy surface structure.

Chemical disinfectants such as chlorine are generally used to control pathogens on fresh produce and have been shown in various studies to be very effective against *L. monocytogenes* (Zang & Farber, 1996). Chlorine treatment of *L. innocua* cells in this study did not result in complete elimination of the organism from the crop. A diminished activity to chlorine has been reported by researchers (Nguyen-the & Carlin, 1994; Albrecht *et al*., 1995; Beuchat *et al*., 1998) and may be due to several factors, such as presence of hydrophobic pockets or folding of the leaf surface, resulting in the creation of environments where the organism is isolated and out of reach of the chemical effect of the sanitizers (Adams *et al*., 1989). When combined with organic material the effect of chlorine may be negated (Beuchat *et al*., 1998), or bacterial resistance to the chemical may play a role (Lisle *et al*., 1998).

Great reliance is placed on post-harvest interventions to limit the number of enteropathogens present on fresh produce at point of sale. In the production of organic produce, however, chemicals cannot be used (Heaton & Jones, 2008).

In analysis of the final results, the possibility of a different heating effect within the plastic bags than in the case of cooking in direct contact with water has to be kept in mind. The present method of cooking might have simulated more closely a steaming effect, as the broccoli florets were contained in a closed package.

Transfer of the contaminated broccoli florets into the 90 ml buffered peptone water in which they were submerged for final microbial analysis, before cooking, "mashing" with the stomacher and cooking this in boiling water in the stomacher bag and subsequent direct analysis from this bag could have been a desirable alternative method. This could have reduced transfer and handling of the contaminated florets and so external influence of factors on the organism survival as well as the possibility of cross contamination. Cooking the florets within this water would also have simulated actual in-water cooking conditions more accurately. Heat transfer would, however, still have been influenced to a degree, however marginal, by the presence of the plastic bag.

Broccoli florets were individually packaged and heated as such during microwave treatment. The lower the quantity of sample being heated, the higher the temperature reached within a given time and also the faster a certain temperature is reached (Ramesh *et al.*, 2002). The small sample size subjected to microwave heating, was therefore responsible for premature sensory deterioration observed, due to higher product temperature reached at a faster cooking time, necessitating the reduction of the cooking time to 30 s, so as to maintain product integrity and so simulate home cooking conditions. In this case, the reduced microwave cooking time did not lead to greater survival of *L. innocua* on the microwaved florets, possibly due to a higher temperature being reached despite the short period of exposure to the heat (Ramesh *et al.*, 2002).

The manner in which the cooking procedure was carried out, prevented comparison of this cooking method with other methods in literature where the cooking action was performed directly in water. The method as performed in the present experiment facilitated a different heating mechanism, as heat transfer did not occur in the water medium, but was collected within the sealed plastic packet. This method of cooking eliminated an additional wash effect during the cooking procedure

and necessitated comparison with a steam effect of cooking rather than with conventional cooking directly in boiling water.

The experimental design could have been widened to enable more conclusive results to be obtained on the individual effects of the different processing steps. The current design performed all processing procedures as steps in a processing chain, preventing the comparison of individual effects with each other and comparison between the effects alone or in combination with one or two other steps, versus as part of the hurdle system.

It has been recognised that the problem of contaminated irrigation water in South Africa is of such a nature that no instant solution to the problem exists. Even though techniques such as filtration, chlorination, ozonation, exposure to ultraviolet light, electronic beam processing and heat treatment can all potentially reduce the levels of microorganisms in irrigation water, the use of these treatments are not practical in many farming situations in South Africa. This is especially so in rural areas. The cost of ensuring high quality irrigation water, however desirable, is also prohibitive (Steele & Odumeru, 2004).

Countries such as France and Germany have a limit of 2 log cfu/g for *L. monocytogenes* in food products (Francis *et al*., 1999), whilst the United Kingdom and the United States, require absence of *L. monocytogenes* on 25g of ready-to-eat food to render it suitable for human consumption (Francis *et al*., 1999). Guidelines published by the South African Department of Health state that *L. monocytogenes* should be absent in one gram of raw vegetable produce (Lötter, 2010).

Due to the fact that *Listeria* is highly ubiquitous, in addition to its virulence, implementing regulations to control the pathogen proves problematic. Realistic and achievable limits for industry have to be implemented, along with ensuring adequate protection for consumers. The zero tolerance enforced in the US, for example, often results in food producers performing minimal monitoring and end-product testing in order to prevent a recall, only assuming that the process hurdles reduce the risk of pathogen incidence. This lack of monitoring is thought to increase the risk that pathogen contamination poses to consumers (Warriner & Namvar, 2009).

In the fresh-cut vegetable industry, ample situations are involuntarily created and opportunities arise for contamination of produce with pathogenic microorganisms. The first risk situation originates from potentially contaminated irrigation water, from where a pathogen, when exposed to the crop surface, may

remain and survive during crop growth in the field to the point of harvest. During harvest and post-harvest processing, wounds and cuts are inflicted on the crops, creating higher nutrient niches for pathogens and increasing opportunity for the pathogen to not only survive, but proliferate. The crops are also exposed to contact of handlers during cutting and packaging, introducing the risk of cross-contamination of vegetables. Hurdles that are implemented to curdle organism survival may not be effective in eliminating the risk of pathogen contamination and sufficient treatment by the consumer after purchase cannot be relied upon to ensure final safety for consumption. The risk posed by the presence of pathogens on fresh vegetables is a reality and necessitates treatment of water and crops as intervention strategies to ensure quality, as well as adequate implementation of critical control points and monitoring at these points to confirm efficacy of processing hurdles. Predictive microbiology is a tool that can be used to support the traditional microbiological methods used to evaluate the potential of *Listeria* growth in foods and the subsequent possibility of foodborne disease (Duh & Shaffner, 1993).Previous quantitative risk assessment models performed for the use of reclaimed water show that risk varies between crops, with lettuce posing a higher risk than cucumber, but comparable to that of broccoli and cabbage (Hamilton *et al*., 2006).

In the year 2009/2010, the per capita consumption of fresh vegetables in South Africa was 42.07kg (Department of Agriculture, Forestry and Fisheries, 2009). According to the data of sales of fresh produce on the country's 20 major fresh produce markets in 2009, broccoli sales made up 0.19% of the total vegetable sales for the year (Department of Agriculture, Forestry and Fisheries, 2009). For purposes of our risk assessment calculation, we thus deduce that the annual per capita consumption of broccoli would be approximately 79.93g.

The U.S Environmental Protection Agency (EPA) set a goal that all water from surface sources should not pose a risk of infection from waterborne pathogens greater than 1:10 000 per year (Stine *et al*., 2005).No experimental dose response data is available on humans for *L. monocytogenes*, meaning that the minimum infective dose of *L. monocytogenes* for humans is unknown. From animal experiments it is known that *Listeria* infections are dose dependent and that the infective dose ($ID_{50}$) is above $10^5$ cells (Schlech III *et al*., 1993; Notermans *et al*., 1998). However, it is not known exactly how to extrapolate these data to humans. To date, a formal risk assessment has not been carried out to establish the relationship between risk of

foodborne listeriosis and the levels of *L. monocytogenes* in various products (Nørrung, 2000). From such a study on the infective dose of *L. monocytogenes*, a median infectious dose can be obtained, as well as a parameter defining the dose response curve. This will then enable the conduction of a risk assessment to determine the concentration of microorganisms that can be present in irrigation water to achieve a specified acceptable risk of infection from vegetable consumption, similar to meeting a food safety objective (Cole, 2004), (Szabo *et al*., 2003). Such a risk assessment has, for example, been carried out for *E.coli* (Powell *et al*., 2000) and Salmonella on lettuce (Stine *et al*., 2005).

$$P_A = 1 - (1 - P_i)^{365}$$

$$(1{:}10\ 000) = 1 - (1 - P_i)^{365}$$

$$P_i(d) = 1 - [1 + (d/N_{50})(2^{1/\alpha} - 1)]^{-\alpha}$$

$$\text{Organisms per gram fresh produce} = \frac{d}{\text{amount fresh produce consumed per person}}$$

$$+ \Sigma R - \Sigma I$$

$$= \text{Final organisms per gram of fresh produce}$$

Concentration of microorganisms in irrigation water to achieve annual acceptable risk

$$= \frac{\text{Final organisms per gram of fresh produce}}{\text{(\% of microorganisms from irrigation water that contaminated surface of produce}}$$

Where:

$P_A$ = Accepted Annual Risk of infection

$P_i$ =Daily acceptable risk

$d$ =Dose of microorganism to achieve calculated $P_i$

$N_{50}$ =Median infectious dose

$\alpha$ = Parameter defining dose-response curve

$\Sigma R$ = Total (cumulative) reduction of hazard

$\Sigma I$ = Total (cumulative) increase of hazard

This leads to the establishment of the maximum concentration of *L. monocytogenes* in irrigation water allowable to achieve an annual acceptable risk of, for example, 1:10 000. Knowledge of a figure such as this would enable the implementation of quality parameters to irrigation water quality in order to ensure the production of fresh produce safe for human consumption.

# CHAPTER 5
# CONCLUSIONS AND RECOMMENDATIONS

## 5.1. CONCLUSIONS

Eliminating pathogens such as *L. monocytogenes* from fresh produce proves to be difficult, as the organisms not only become established on the surface of the produce, but are able to survive post-harvest treatments such as disinfectants and even heat treatment. Prevention of contamination is preferable and an improved understanding of the behaviour of enteropathogenic bacteria on vegetables could assist in the elimination of this contamination. Ensuring irrigation water of good microbial quality is desirable, but as this is not always possible, pre- and post-harvest treatment of crops is warranted.

From the results obtained by the use of *L. innocua* as surrogate organism, it was concluded that irrigation with water containing high *L. monocytogenes* numbers results in an elevated load of the pathogen on broccoli immediately after irrigation, suggesting possible pathogen transfer from the water to the vegetable. Continued application of such contaminated water on broccoli contributes to maintenance of *L. monocytogenes* on broccoli pre-harvest, whilst cessation of irrigation results in significant reduction of the pathogen, making this a possible method to eliminate pathogen presence on the crop at harvest and subsequently at point of sale. Applying irrigation water containing high numbers of *L. monocytogenes* further contributes to an elevated total microbial load on broccoli.

Washing broccoli with chlorinated water (200 ppm) reduces *L. monocytogenes* numbers on broccoli more than washing with unchlorinated water, but does not eliminate the pathogen from the crop surface. Cooking broccoli succeeds in reducing *L. monocytogenes* numbers on broccoli, whilst microwave heating is lethal to the pathogen. In this study, combined treatments of washing with chlorine, storage under conditions of modified atmosphere (MAP: 5% $CO_2$, 5% $O_2$) and reduced temperature (4°C) post-harvest and before consumption, with final microwave heating resulted in the lowest pathogen numbers. Minimal processing procedures can therefore be more effective in preventing *L. monocytogenes* survival on broccoli up to point of sale when implemented as part of a hurdle system.

*L. monocytogenes* has been observed to survive, albeit in low numbers, on broccoli. Vegetables, can, however, still be considered of a lower risk for listeriosis than certain other foods because of its inability to support the extended growth of *L. monocytogenes* (Flessa *et al*., 2005) in comparison to other leafy vegetables that are eaten raw more frequently, or foods such as dairy products that are richer in and provide more accessible growth-supporting nutrients.

## 5.2. RECOMMENDATIONS

Further information on the factors which influence pathogen survival in the field may enable the development of new and improved intervention strategies to control bacterial persistence on fresh produce.

Research into the exact mode and conditions of attachment of *L. monocytogenes* to different vegetable surfaces, along with studies into the mechanism of destruction or even survival could prove to be valuable. Microscopic studies on membrane integrity accompanying the cell counts could indicate the mode and sights of injury where the organisms are influenced by certain extreme conditions and treatments. Pathogens might also be able to respond to sublethal stresses by entering a survival mode, enabling them to recover once favourable conditions are restored.

Studies into the possibility and method of internalisation of the pathogen into the crops could provide insight into the hidden risk associated with certain vegetables. Once internalised, a pathogen is inaccessible by means of washing techniques or chemical disinfectants (Milillo *et al*., 2008). For pathogens to infiltrate into fruit and vegetable tissues, water pressure on the produce surface has to exceed internal gas pressure and the hydrophobicity of the produce surface has to be overcome (Beuchat, 2002).

The incidence of viable but non-culturable (VBNC) organisms exists as they adapt to conditions of stress by various mechanisms of survival (Byrd *et al*., 1991). The occurrence of *L. monocytogenes* cells entering this state upon application of environmental stresses should be taken into account when determining cell counts of pathogens on vegetables in the field and post-harvest.

Future research is needed to determine if the growing season affects vegetable contamination. It has also been suggested that a correlation between environmental

conditions be used to determine an optimal harvesting time when contamination is at its lowest (Prazak *et al*., 2002b).

An extended shelf-life study under different packaging atmospheres and storage temperatures would bring forward practical information on the long-term effect of processing on the pathogen. The possibility exists that a pathogen might have entered a viable but non-culturable state after processing or have a stress-response and is able to recover with time elapsed. Greater insight into the survival of the pathogen under different concentrations of modified atmosphere would also be valuable.

Because treatment options are limited, it is better to prevent contamination of surface water. Controlling surface water contamination from nonpoint sources, such as birds and wildlife, is extremely difficult. The effect of other sources of contamination, such as manure used as fertiliser and runoff from feedlots, can be reduced by following good agricultural practices (GAP) during growth of crops and by employing good hygiene practices (GHP) during harvesting and post-harvest processing (Prazak *et al*., 2002b). GAP includes keeping irrigation sources away from livestock such as cows and poultry, identifying upstream uses of surface waters that are used for irrigation, such as streams and rivers and ensuring that manure applied to fields does not run into irrigation sources (Rangarajan *et al*., 2003).

Apart from improving the microbial quality of irrigation water before its application, several other strategies can reduce the risk of disease transmission from pathogenic microorganisms on fruit and vegetables. These include restricting the use of poor-quality irrigation water to crops that are not likely to be consumed raw and irrigating with lower quality water early in the growing season and with water of better quality closer to harvest, therefore relying on pathogen die-off before harvest. Using drip irrigation rather than spray irrigation has also been suggested to ensure less microbial contamination of crops (Steele & Odumeru, 2004). Postharvest washing of fruits and vegetables remains the most common treatment to reduce the risk of pathogen transmission from vegetables to consumers. Pathogens can, however, escape the effect of disinfectants in crevices and harvest trimming wounds (Steele & Odumeru, 2004) and controlling the hazard through the implementation of HACCP and a Standard Sanitation Operating Procedures (SSOP) is a necessity (Warriner & Namvar, 2009). Prevention of initial contamination remains preferable (Steele & Odumeru, 2004).

Further research into the behaviour of pathogens on and their interaction with the plant environment as well as the factors that can limit their survival in the field will assist in developing improved agricultural practices for pre- and post-harvest safety of fresh vegetables.

A quantitative risk assessment of the risk of disease from pathogens present in water used to irrigate crops would be a beneficial area of further research.

The development of a database of PCR fingerprints to trace cases of listeriosis to certain production areas has been suggested to gain greater control over outbreaks of foodborne illness.

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