ISOLATION AND ANTIMICROBIAL SUSCEPTIBILITY CHARACTERISATION OF *LISTERIA* SPP. IN SELECTED FOOD PREMISES IN CENTRAL SOUTH AFRICA

MARINA J SNYMAN

Dissertation submitted in fulfillment of the requirement for the Degree:

MAGISTER TECHNOLOGIAE:

ENVIRONMENTAL HEALTH

in the

FACULTY OF HEALTH AND ENVIRONMENTAL SCIENCES

at the

CENTRAL UNIVERSITY OF TECHNOLOGY, FREE STATE

Supervisor: Dr MM Theron

Co-supervisors: Prof JFR Lues

Dr O de Smidt

BLOEMFONTEIN

2011

DECLARATION OF INDEPENDENT WORK

I, MARINA J SNYMAN, hereby declare that the dissertation submitted for the degree MAGISTER TECHNOLOGIAE in ENVIRONMENTAL HEALTH, at the Central University of Technology, Free State, is my own original work and has not previously been submitted to any other institutions by myself or any other person in fulfillment of the requirements for the attainment of any qualification.

……………………………….
SIGNATURE OF STUDENT

……………………….
DATE

ACKNOWLEDEMENTS

I would like to thank the following people and institutions:

Dr MM Theron for her patience, all her expertise, encouragement, assistance and time during the study and with the editorial assistance;

Dr O de Smidt for her guidance, assistance and time during the study;

Prof JFR Lues for his enthusiasm;

My husband and daughters for all their love, encouragement and prayers;

My friends and colleagues for their support;

The Unit of Applied Food Science and Biotechnology for the use of the laboratory facilities;

The Central University of Technology for financial assistance;

The National Research Foundation for all the financial support.

Finally: “It is choice – not chance – that determines your destiny”; Jean Nidetch.

SUMMARY

Microbial pathogens play an important role in the food industry where they could cause disease and subsequently significant economic losses. Limited information is available on the situation with regard to *Listeria* in food products in South Africa. However, much research is being done in the rest of the world on *Listeria* indicating serious problems as a result of resistance development against various antimicrobial agents, including the organic acids. It is hypothesised that the situation with regard to resistance development may be more serious than generally admitted. Isolation of 200 different food samples was done by using a slightly modified EN ISO 11290-1/A1:2004 standard method. Identification of presumptive positive colonies was confirmed as *Listeria* by API (Analytical profile index) *Listeria*. API positive cultures were subjected to 16S rDNA sequencing to compare and confirm identification. Isolates and standard strains were screened for resistance to food preservatives such as organic acids and antibiotics used in the current treatment regime for *Listeria* infections. The organisms evaluated included isolated strains namely *Listeria monocytogenes, Listeria welshimeri, Listeria innocua* and their corresponding ATCC (American type culture colletion) strains. An agar dilution method as described by the Clinical and Laboratory Standard Institute (CLSI) was used to determine the minimum inhibitory concentrations (MICs) of 11 antibiotics and 13 organic acids and salts for all the isolates. Overall antibiotic susceptibility patterns of all the isolates indicated high level susceptibility to all the antibiotics tested. Susceptibility to all the organic acids was notably reduced at pH 7 in all the isolates and control strains.

Eight highly susceptible strains were selected for induction and represented each of the species isolated. These isolates were exposed to increasing concentrations of three antibiotics and three organic acids. MICs were again determined for all the induced strains for five antibiotics and three organic acids. Proteins extracted from the induced strains were separated on discontinuous SDS-PAGE slab gels to generate total protein profiles. Notable variations were observed in MICs, although induction with antibiotics as well as organic acids did not result in general resistance development. However, evidence was provided that continuous exposure to antimicrobial agents may cause *Listeria* spp. to develop resistance to different antimicrobial agents. Further research and in depth studies on mechanisms involved in the development of resistance to food preservatives would, therefore, be required.

Finally, it is concluded that *Listeria monocytogenes* may be a possible threat in the Central South African food industry, which deserves more attention. The situation may actually pose a problem that is overseen, because only a small percentage of people that get sick from food, would seek medical advice.

LIST OF FIGURES

FIGURE	TITLE	PAGE
2.1	Diagrammatic representation of the method followed for detection of *Listeria* spp.	39
2.2	Agarose gel electrophoresis of *iap* gene products amplified from control *Listeria* genomic DNA	52
2.3	Agarose gel electrophoresis of *iap* gene products amplified from control *Listeria* genomic DNA after PCR optimisation	52
3.1	Comparison of MICs obtained at two pH values (pH7 and pH5) for propionic acid	73
4.1	Total protein profiles of seven *Listeria* spp. after induction with various antibiotics	99

LIST OF TABLES

TABLE	TITLE	PAGE
1.1	Food-borne outbreaks of invasive listeriosis with ten or more cases	7
2.1	Potential selective reagents for enhancement of *Listeria* isolation	32
2.2	API profiles of presumptive *Listeria* strains isolated from food and dairy samples	46
2.3	*Listeria* isolates identified using API and 16S rDNA sequencing	47
2.4	*Listeria monocytogenes* detection limits in various food products	53
3.1	Antibiotics included in MIC determination	65
3.2	Antibiotic susceptibility ranges for *Listeria innocua* isolates compared to the control strain	68
3.3	Antibiotic susceptibility ranges for *L. monocytogenes* isolates compared to the control strain	69
3.4	Antibiotic susceptibility ranges for *Listeria welshimeri* isolates compared to the control strain	70
3.5	Susceptibility of *Listeria monocytogenes* isolates to organic acids compared to the control strain	75
3.6	Susceptibility of *Listeria welshimeri* isolates to organic acids compared to the control strain	76
3.7	Susceptibility of *Listeria innocua* isolates to organic acids compared to the control strain	77

4.1 Comparison of inhibitory concentrations of five antibiotics and three organic acids after induction of three *Listeria monocytogenes* and *L. monocytogenes* ATCC19111. 94

4.2 Comparison of inhibitory concentrations of five antibiotics and three organic acids after induction of four *Listeria welshimeri* and *L. welshimeri* ATCC 35897 95

4.3 Comparison of inhibitory concentrations of five antibiotics and three organic acids after induction of one *Listeria innocua* and *L. innocua* ATCC 33090 96

TABLE OF CONTENTS

		PAGE
CHAPTER 1		
Literature review		**1**
1.1	Introduction	2
1.2	Identification of *Listeria*	3
1.3	*Listeria* from the environment	4
1.4	*Listeria* in food	6
1.5	Clinical manifestation of *Listeria monocytogenes*	8
1.6	Susceptibility patterns of *Listeria*	9
1.7	Impact of organic acids on *Listeria* in food preservation	11
1.8	*Listeria* in Africa	12
1.9	Antimicrobial resistance in *Listeria*	13
1.10	Prevention of *Listeria* contamination	15
1.11	Rationale	15
1.12	References	17
CHAPTER 2		
Isolation and Identification of *Listeria* spp. from various food premises in Central South Africa		**29**
2.1	Introduction	30
	2.1.1 Selective media for enrichment and isolation of *Listeria*	32

2.1.2 Rapid methods for detection of *Listeria* 34

2.1.3 Polymerase chain reaction (PCR) 34

2.2 Materials and Methods 35

2.2.1 Sampling methods 35

2.2.2 Culturing procedures 37

2.2.3 DNA based methodologies 40

2.2.3.1 Template preparation 40

2.2.3.2 PCR set up conditions 42

2.2.3.3 DNA sequencing 44

2.3 Results and Discussion 45

2.3.1 Isolation 45

2.3.2 DNA based results 50

2.4 Conclusions 54

2.5 References 56

CHAPTER 3

Comparing antibiotic and organic susceptibility in *Listeria* spp. 62

3.1 Introduction 63

3.2 Materials and Methods 64

3.2.1 Antibiotics 64

3.2.2 Organic acids 66

3.3 Results and Discussion 67

3.3.1 Antibiotic susceptibility 67

3.3.2 Organic acid susceptibility 72

3.4 Conclusions 80

3.5 References 82

CHAPTER 4

Exposure of *Listeria* spp. to Sub-lethal Treatment of Antimicrobial Agents **87**

4.1 Introduction 88

4.2 Materials and Methods 90

4.2.1 Induction 90

4.2.2 Susceptibility Testing 90

4.2.3 Protein Extraction 91

4.3 Results and Discussion 93

4.4 Conclusions 101

4.5 References 102

CHAPTER 5

General Discussion **103**

5.1 Background 104

5.2 Isolation and Identification of *Listeria* 105

5.3 Antimicrobial Susceptibility of *Listeria* 105

5.4 Inducing Antimicrobial Resistance 107

5.5 Future Research 108

5.6 References 110

APPENDIX A **111**

A.1 Antibiotic susceptibility of *Listeria innocua* isolates 112

A.2 Antibiotic susceptibility of *Listeria monocytogenes* isolates 113

A.3 Antibiotic susceptibility of *Listeria welshimeri* isolates 114

A.4 Susceptibility of *Listeria monocytogenes* isolates to organic acids (n=6) at pH 5 115

A.5 Susceptibility of *Listeria monocytogenes* isolates to organic acids (n=6) at pH 7 116

A.6 Susceptibility of *Listeria welshimeri* isolates to organic acids (n=36) at pH 5 117

A.7 Susceptibility of *Listeria welshimeri* isolates to organic acids (n=36) at pH 7 118

A.8 Susceptibility of *Listeria innocua* isolates to organic acids (n=8) at pH 5 119

A.9 Susceptibility of *Listeria innocua* isolates to organic acids (n=8) at pH 7 120

APPENDIX B **121**

B.1 Total protein profiles of *Listeria monocytogenes* 60 after induction with various antimicrobial agents 122

B.2 Total protein profiles of *Listeria monocytogenes* 138 after induction

with various antimicrobial agents 123

B.3 Total protein profiles of *Listeria monocytogenes* 139 after induction with various antimicrobial agents 124

B.4 Total protein profiles of *Listeria monocytogenes* ATCC 19111 after induction with various antimicrobial agents 125

B.5 Total protein profiles of *Listeria welshimeri* 87 after induction with various antimicrobial agents 126

B.6 Total protein profiles of *Listeria welshimeri* 88 after induction with various antimicrobial agents 127

B.7 Total protein profiles of *Listeria welshimeri* 92 after induction with various antimicrobial agents 128

B.8 Total protein profiles of *Listeria welshimeri* 95 after induction with various antimicrobial agents 129

B.9 Total protein profiles of *Listeria welshimeri* ATCC 35897 after induction with various antimicrobial agents 130

B.10 Total protein profiles of *Listeria innocua* 105 after induction with various antimicrobial agents 131

B.11 Total protein profiles of *Listeria innocua* ATCC 33090 after induction with various antimicrobial agents 132

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Listeria was initially included among the coryneform bacteria and actinomycetes (Bousfield, 1972). In 1969 *Listeria* was compared to various representatives of lactic acid bacteria (Davis et al., 1969). In 1988 *Listeria* was distinguished from other known genera, including *Erysipelothirix* and *Brochothrix thermosphacta* and recognised to be related to *Lactobacillus* and *Streptococcus* (Freesu and Jones, 1988). After 25 years of studies by various laboratories in different countries, the genus *Listeria* currently contains six species; *Listeria monocytogenes, L. ivanovii, L. innocua, L. welshimeri, L. seeligeri* and *L. grayi* (Rocourt and Buchrieser, 2007). *L. monocytogenes* is recognised as a human pathogen, although *L. seeligeri* and *L. ivanovii* have also been implicated in human infections (McLauchlin, 1996). The distinction between the pathogenic *L. monocytogenes* and the other nonpathogenic species was already defined when food-borne listeriosis became a public health problem in the United States and Europe.

L. monocytogenes is a Gram-positive non-spore forming bacillus and occurs widely in agricultural (soil, silage, water, faecal material, sewage) and food processing environments. *L. monocytogenes* is also a resident of the intestinal tract in humans resulting in carriers of the microorganism without any apparent health consequences. In comparison to other non-spore forming food-borne pathogenic bacteria (e.g. *Salmonella spp*. and enterohemorrhagic *Escherichia coli*), *L. monocytogenes* is resistant to environmental conditions such as acidity or high salt. This bacterium grows at low refrigeration temperatures (2-8°C) with low oxygen levels and survives for long periods

in the environment. Listeriosis is generally associated with ready-to-eat, refrigerated foods and often involves the post-processing recontamination of cooked foods. Low levels of *L. monocytogenes* in contaminated food may multiply during storage in foods that support growth, even at refrigeration temperatures (2-8°C). Invasive listeriosis is relatively rare, but often severe disease with incidences of 3 to 8 cases per 1,000,000 individuals reported world wide with fatality rates of 20 to 30% among hospitalised patients (Food and Agriculture organisation of the United Nations/World Health Organisation food standards program, 2005).

Statistics of the incidence of food-borne diseases in the Central Region of South Africa caused by the genus *Listeria* are limited and only a few articles are available in South Africa on studies done that include this organism.

1.2 IDENTIFICATION OF *LISTERIA*

Listeria is a Gram-positive, non-spore forming, facultative anaerobic, motile bacillus. The organism is catalase positive and oxidase negative and the tumbling effect of the active motility characteristic of this bacterium can be observed with a light microscope. Motility is visible when cultured at 20 to 25˚C (Rocourt and Buchrieser, 2007). After enrichment in a broth, colonies are isolated on selective agar. The most commonly used pre-enrichment and selective enrichment broth is Fraser Broth. *Listeria* will turn Fraser broth black from esculin hydrolysis within 48 h of incubation (Buchanan, 1988).

Selective agar includes Oxford agar, Palcam agar and *Listeria* agar according to Ottaviani et al. (1997). These media all contain supplements and inhibition substances for “non-*Listeria”* flora. Selective agents include acriflavin (to inhibit various Gram-positive bacteria) and nalidixic acid (to inhibit all Gram-negative bacteria).

1.3 *LISTERIA* FROM THE ENVIRONMENT

The organism that was originally named *Listerella hepatolytica* was first discovered in 1927 by Pirie while investigating the unusual deaths of field rodents near Gauteng in South Africa (Rocourt and Buchrieser, 2007). *Listeria* has been implicated in diseases affecting both man and animals. It was initially believed that farm animals transmitted *L. monocytogenes* to farm workers as the organism has specifically been isolated from many different environments including soil, water, vegetation, sewage, animal feeds, farm environments, and food-processing environments (Saunders and Wiedmann, 2007). Faecal contamination of vegetables from farm animals, due to improperly fermented silage, may result after fertilisation with animal manure. Listeriosis started to appear in people living in the city, and it was only then that public health authorities realised that animal contact was not always the source of disease transmission (USDA/FSIS, 1992).

In 1983 a food-borne outbreak of human listeriosis was associated with coleslaw from crops that were fertilised with contaminated sludge. A variety of food products is

produced from farm animals that can be contaminated with *Listeria* spp. (UDSA/FSIS, 1992), and caution should therefore be taken when applying animal wastes on crops that may be consumed raw (Schlech et al., 1983).

An investigation by Garrec et al. (2003) on the impact of sludge treatments in different treatments plants showed that liming of sludge was found to reduce *Listeria* spp. loads to less than detectable levels. It was also found that 73% of dewatered sludge contained *L. monocytogenes* and 87% contained *Listeria* spp. Moreover, 96% of sludge stored in tanks contained *Listeria* spp. and 80% contained *L. monocytogenes.* From Italy it was reported that the highest concentrations of *Listeria* spp. (*L. monocytogenes*, *L. innocua, L. welshimeri*, and *L. grayi*) were isolated from activated sludge that was fertilised (De Luca et al., 1998).

In various studies from different countries, *Listeria* spp. had been found in a wide range of surface waters, including lakes, rivers and streams. *L. monocytogenes* has been reported to be present in varying numbers in water (Watkins and Sleath, 1981; Fenlon et al., 1996). Studies done in a lake in Greece showed the prevalence of *L. monocytogenes* to be only 4% (Arvanitidou et al., 1997) and in Belgium a smaller survey of 15 groundwater samples found *L. monocytogenes* in only one sample (Van Renterghem et al., 1991). There is however, no epidemiological data available which show that listeriosis was caused by water contaminated with *L. monocytogenes* (Sauders and Wiedmann, 2007).

Domestic animals such as sheep, cattle, goats and birds are all susceptible for listeric infection, and a number of healthy asymptomatic animals may shed *L. monocytogenes* in their faeces. Animals usually become infected with *L. monocytogenes* by ingestion of contaminated feed (Wesley, 2007) which subsequently colonises the medulla oblongata, viscera or gravid uterus (Kimberling, 1988). Although livestock management practices as well as climate differ in countries, listeriosis in domestic livestock is recognised worldwide (Doyle, 1994). For example, in the Netherlands annual livestock losses have been reported to be between 0.7 and 8.7% with an average of 3.2%. From 1970 to 1985 losses of between 234 and 928 cases occurred as a result of bovine abortions attributed to *L. monocytogenes* in cattle (Dijkstra, 1987). Transmission of *L. monocytogenes* from livestock to humans occurs by direct contact with infected animals, especially during lambing or calving and also the consumption of contaminated raw milk (Sauders and Wiedmann, 2007).

1.4 *LISTERIA* IN FOOD

Currently the most common cause of human listeriosis infections in the United States is reported to be the consumption of *L. monocytogenes* contaminated ready-to-eat (RTE) meat products (FDA/USDA/CDC, 2003). RTE products permit growth of *L. monocytogenes* because of its ability to grow at refrigeration temperatures. Norton and Braden (2007) reported that the highest incidence of recalls for cooked and RTE meat products contaminated with *L. monocytogenes* in the United States from 1991 to

November 2006 was 35 000 000 lbs of hot dogs and packaged meat. In Table 1.1 the highest worldwide incidence of reported invasive listeriosis outbreaks from 1945 to 1999 and the associated foodstuff are illustrated.

TABLE 1.1: Food-borne outbreaks of invasive listeriosis with ten or more cases

YEAR	LOCATION	SUSPECT/IMPLICATED FOOD	NO. CASES[a]
1945-1952	Halle, East Germany	Raw milk, sour milk, cream, cottage cheese	100
1960-1961	Bremen, West Germany	Unknown	81
1966	Halle, East Germany	Unknown	279
1975-1976	Anjou, France	Unknown	162
1983-1987	Vaud, Switzerland	Vacherin Mont d'Or cheese	122
1985	Los Angeles	Mexican-style cheese	142
1987-1989	England, Wales, North Ireland	Pâté	366
1992	France	Pork tongue in aspic (jelly)	279
1998-1999	United States, multistate (n = 24)	Processed meats	108

[a]Number of cases reflects laboratory-confirmed and epidemiologically linked cases (Norton and Braden, 2007)

Outbreaks of listeriosis have also been linked to seafood items. These include imitation crab meat, smoked mussels, gravad (salmon), and cold-smoked fish. Few listeriosis outbreaks have been linked to consumption of contaminated vegetables or vegetable products. However, in 1981 during an investigation of a large epidemic of listeriosis in adults and perinatals in the Maritime Provinces of Canada, officials hypothesised that cabbage, contaminated with *L. monocytogenes,* was the causative agent (Schlech et al., 1983). The short shelf-life of unpreserved vegetable products may contribute to the infrequent association with invasive disease.

1.5 CLINICAL MANIFESTATION OF *LISTERIA MONOCYTOGENES*

L. monocytogenes has been identified to be the causative agent of listeriosis in humans (Bille, 1990; Gilbert et al., 1993; Jacquet et al., 1995). *L. monocytogenes* is also a food-borne pathogen and approximately 10% of all deaths related to food-borne illnesses in the United States are caused by this pathogen (Mead et al., 1999). Listeriosis is a disease contracted after eating food contaminated with this organism. People that are particularly at risk include pregnant women as infection may lead to spontaneous abortions or serious illness in newborns. The highest incidence of listeriosis has been in persons over 60 years old as well as newborns. Immune-compromised patients such as those suffering from cancer and AIDS or patients using immunosuppressive medication such as steroids are also at risk. Other patients include those suffering from cirrhosis, diabetes and ulcerative colitis (USDA/FSIS, 1992).

Symptoms of listeriosis may vary and depend on the individual's susceptibility. Early symptoms include fever, fatigue, nausea, vomiting and diarrhea. These symptoms may become more serious and can result in meningitis (brain infections) and septicemia (bacteria in the bloodstream). Severe listeriosis in pregnant women, starting with flu-like symptoms, can result in miscarriage, stillbirth, septicemia or meningitis in the newborn. In adults and older children complications usually affect the bloodstream and central nervous system, but may also include pneumonia and endocarditis (inflammation of the lining of the heart and valves). Skin contact with *L. monocytogenes* can cause skin

lesions or localised abscesses. Flu-like symptoms may occur 12 hours after eating contaminated food while it takes from one to six weeks for a serious case of listeriosis to develop (USDA/FSIS, 1992).

1.6 SUSCEPTIBILITY PATTERNS OF *LISTERIA*

Many pathogens, including *Listeria,* are developing resistance to most antibiotics used in current treatment regimes, with frequent reports of pathogens being resistant to almost all available antibiotics (Levy, 1998). Antibiotic resistance is increasingly widely reported in all bacteria, mainly as a result of the over-use of antibiotics in animals and humans (Davies, 1998; Rao, 1998). Transfer of genetic material, carrying resistance determinants, is also possible between unrelated bacterial species (Kruse and Sorum, 1994). Many antibiotic-resistant bacteria in foods are saprophytic or commensal habitants but their resistance genes can be transferred to other food-borne bacteria. These include pathogenic species found in the gastrointestinal tract (Perreten et al., 1997). This process can have clinical implications for the host and for the wider population that comes into contact with antibiotic-resistant pathogens.

Listeriosis has commonly been treated with penicillin or ampicillin in combination with an aminoglycoside (Charpentier and Courvalin, 1999), while alternative treatments included tetracycline, erythromycin or chloramphenicol, alone or in combination (Hof, 1991). Currently a combination of ampicillin and gentamicin is standard therapy for

systemic listeriosis, and trimethoprim-sulfamethoxazole (TMP-SMX) may be used for patients with beta-lactam intolerance (Schlech, 2000). Although *Listeria* spp. have been reported to be susceptible to antibiotics (Hof, 1991), more recent reports have indicated resistance in *Listeria* spp. to antibiotics active against Gram-positive bacteria (Abrahim et al., 1998). This development of antibiotic resistance among *Listeria* spp. shows a similar pattern world-wide and is on the increase (Walsh et al., 2001).

In a study by Walsh et al. (2001) susceptibility of 1001 *Listeria* strains isolated from 67 retail food samples was determined for eight antibiotics. The antibiotics included tetracycline, penicillin G, ampicillin, streptomycin, erythromycin, vancomycin, chloramphenicol and gentamicin. Of the isolates, 10.9% displayed resistance to one or more antibiotics. Resistance to one or more antibiotics was found in 0.6% of *L. monocytogenes* isolates compared to 19.5% of *L. innocua* isolates, and no resistance in *L. seeligeri* or *L. welshimeri.* Resistance to tetracycline (6.7%) and penicillin (3.7%) was also prevalent.

Resistance to antibiotics most commonly used to treat human listeriosis was not observed in *L. monocytogenes,* but the presence of such resistance in other *Listeria* spp. raises the possibility of future acquisition of resistance by *L. monocytogenes.* In addition, the higher level of resistance in *L. innocua* as opposed to that of *L. monocytogenes* suggests the existence of a species related ability to acquire resistance to antibiotics (Walsh et al., 2001; Morvan et al., 2010).

1.7 IMPACT OF ORGANIC ACIDS ON *LISTERIA* IN FOOD PRESERVATION

There is an increasing demand for natural processed food in the food industry. This has caused a growing interest in naturally produced antimicrobial agents including organic acids (McEntire et al., 2003). Because of their natural origin and preservative, antioxidant, flavouring and acidifying properties, as well as low cost, organic acids and their salts are widely used as preservatives in the production of various food products (Crozier-Dodson et al., 2005).

The treatment of fresh meat with organic acids can provide other means of extending distribution and visual attraction (Bauernfeind and Pinkert, 1970; Barker and Park, 2001; Haung et al., 2005) while cured meats such as sausage, ham, and frankfurters already contain salt and other preservatives which enhance the listericidal effects of organic acids (Doyle, 1999). Organic acid(s) present in acidified food products, for example apple cider, dry-fermented sausage, mayonnaise and yoghurt, have made a huge contribution to the safe production of these products without heat treatments (Zagory and Garren, 1999).

L. monocytogenes has been isolated from fresh and minimally processed vegetables (Francis and O'Breine, 2006). However, the application of washing solutions containing bacteriocins (produced by lactic acid bacteria), to fresh-cut lettuce has been reported to inhibit the proliferation of *L. monocytogenes* during storage (Allende et al., 2007). Consumption of unpasteurised fruit juices has increased in recent years because of

freshness, high vitamin content as well as low calorie contribution (Harris et al., 2003). However, the incidence or survival of *L. monocytogenes, L. innocua, Salmonella* serovars and *Escherichia coli* in fruit juices and apple cider has been demonstrated (Ceylan et al., 2004). Different concentrations of malic acid added to apple, pear and melon juices have been demonstrated to inactivate these pathogens by more than 5 log cycles after 24 h storage at 5˚C (Raybaudi-Massilia et al., 2009).

1.8 *LISTERIA* IN AFRICA

A limited number of studies have been done on the prevalence of *Listeria* spp. in African food products. African countries where such studies have been done include Morocco, Nigeria, Ghana, Egypt, Ethiopia and Senegal (Ababouch, 2000). Research carried out in Morocco showed an increase in the incidence rate, with 10.5-86.3% involving meat products (Kriem et al., 1998) and 10-18% involving raw milk, fermented milk-based products and fresh cheese (El Marrakchi et al., 1993).

In South Africa, however, there are few statistics available on the prevalence of *Listeria* found in food such as RTE meat, poultry, milk and soft cheeses. A study done by Vorster et al. (1993) in South Africa on the incidence of *Listeria* in 134 retail samples of processed meats, showed only 8% overall prevalence in Vienna sausage, ham, and cervelat. *L. monocytogenes* was not found in any of these samples. More recently Van Nierop et al. (2005) conducted a survey on the microbial quality of 99 fresh and frozen

chicken carcasses from butchers, supermarkets and street vendors in Gauteng. Of the 99 carcasses 19 (19.2%) were culture positive for *L. monocytogenes,* but PCR amplification showed 41 of 99 carcasses positive for *L. monocytogenes*. Sixty six fresh and 33 frozen carcasses were tested, of which 19.2% and 21.2% rinsed samples respectively yielded *L. monocytogenes*. The highest contamination rate was seen on frozen carcasses from butchers (35.3%) and the lowest rate from supermarkets (6.3%). One out of six chickens slaughtered and plucked by street vendors was found to be infected with *L. monocytogenes*.

1.9 ACID TOLERANCE IN *LISTERIA*

The effect of organic acids used as preservatives in food production is not always positive in terms of food safety. *Listeria,* which survive after exposure to these acids may repair themselves during storage at low temperatures and continue to multiply (Cheroutre-Vialette et al., 1998). Exposure to acid also induces stress responses in listeriae which make the bacteria more tolerant of more acidity (Lou and Yousef, 1997).

Various studies have shown that *L. monocytogenes* is more acid tolerant than most food-borne pathogens, although sensitivity of the organism to organic acids varies with the nature of the acidulant used (Sorrells et al., 1989). Acid tolerance is enhanced by exposing the organism to moderately acidic conditions (Davis et al., 1996; Kroll and Patchett, 1992). This is of importance as *L. monocytogenes* often encounters a low pH

environment in acidic foods and during gastric passage in the host (Gandhi and Chikindas, 2006).

The optimum pH for *L. monocytogenes* growth is 7 to 8, but this organism may grow in a pH range of 5 to 10 (Sorrells et al., 1989). However, Barker and Park (2001) have shown that this organism can survive and grow at a pH as low as 4.4. To tolerate salt stress *Listeria* may change its gene expression, which leads to an increased or decreased synthesis of various proteins. In response to salt stress, *L. monocytogenes* has been reported to induce 12 proteins (Duche et al., 2002). When confronted with acidic conditions, *Listeria* cells resist adversity by increasing the synthesis of proteins which participate in the resistance mechanisms. When exposed to a more severe acidity, *Listeria* attempt to resist further by synthesising additional stress proteins (Phan-Thanh, 2000).

Another survival mechanism of *Listeria* spp. is the ability of the organism to grow over a wide range of temperatures (2–45˚C). Refrigeration is one of the most common ways to increase the shelf life of foods, and the survival and growth of *L. monocytogenes* at refrigeration temperatures (2–4˚C) are, therefore, two of the many factors that complicate the control of this food-borne pathogen (Rocourt and Cossart, 1997).

1.10 PREVENTION OF *LISTERIA* CONTAMINATION

Recognition that most human listeriosis is food-borne has led to control measures that have reduced the incidence of listeriosis. In 2001 and 2003, the Food and Drug Administration (FDA), Centre for Disease Control (CDC) and the United States Department of Agriculture (USDA) released a national *Listeria* Action Plan in the United States to control efforts by industry, regulators and public health officials, to adhere to the zero-tolerance policy of the US food industry (FDA/CDC, 2003). However, control of listeriosis requires action from public health agencies as well as the food industry (Elliot and Elmer, 2007). The food industry must understand how contamination occurs and then implement hazard analysis critical control point (HACCP) programs to minimise the presence of *L. monocytogenes* at important points in the processing, distribution and marketing of processed foods (Anonymous, 1991). According to the Regulations Governing Microbiological Standards For Foodstuffs and Related Matters in South Africa no provision has been made for microbiological specifications with regards to *Listeria monocytogenes* (South African Department of Health, 2001).

1.11 RATIONALE

Although much research is being done world-wide on *Listeria* indicating serious problems as a result of resistance development against various antimicrobial agents, including organic acids, limited information is available on the situation with regard to

Listeria in food products in South Africa. Ultimately, this study endeavours to contribute to the body of knowledge with regard to the situation.

The aims of this study, therefore, are:

- To determine the presence of various *Listeria* strains from various food premises and abattoirs in the Free State province.
- To identify isolates by using prescribed methods and selective culture media.
- To screen isolates and reference strains for resistance to food preservatives such as organic acids and antibiotics used in the current treatment regime for *Listeria* infections.
- To determine the possibility of cross-resistance against antibiotics and organic acids, and
- To determine possible mechanisms involved in resistance development against antibiotics as well as the organic acids.

1.12 REFERENCES

Ababouch, L. 2000. Potential of *Listeria* hazard in African fishery products and possible control measures. *International Journal of Food Microbiology***,** 62:211-215.

Abrahim, A., Papa, A., Soultos, N., Ambrosiadis, I. and Antoniadis, A. 1998. Antibiotic resistance of *Salmonella* spp. and *Listeria* spp. isolates from traditionally made fresh sausages in Greece. *Journal of food protection*, 61:1378-1380.

Allende, A., Martinez, B., Selma, V., Gil, M.I., Suárez, J.E. and Rodriguez, A. 2007. Growth and bacteriocin production by lactic acid bacteria in vegetable broth and their effectiveness at reducing *Listeria monocytogenes in vitro* and in fresh-cut lettuce. *Food Microbiology***,** 24:759-766.

Anonymous. 1991. *Listeria monocytogenes*: recommendations by the National Advisory Committee on microbiological criteria for foods. *International Journal of Food Microbiology***,** 14:186-246.

Arvanitidou, M., Papa, A., Constantinidis, T.C., Danielides, V. and Katsouyannopoulos, V. 1997. The occurrence of *Listeria* spp. and *Salmonella* spp. in surface waters. *Microbiological Research***,** 152:395-397.

Barker, C and Park, S.F. 2001. Sensitization of *Listeria monocytogenes* to low pH, organic acids, and osmotic stress by ethanol. *Applied and Environmental Microbiology,* 67:1549-1600.

Bauernfeind, J.C. and Pinkert, D.M. 1970. Food processing with added ascorbic acid. *Advances in Food Research***,** 18:219-315.

Bousfield, I. 1972. A taxonomic study of some coryneform bacteria. *Journal of General Microbiology,* 71:44-455.

Bille, J. 1990. Epidemiology of human listeriosis in Europe, with special reference to the Swiss outbreak. In *Food- borne listeriosis***,** eds. A.J. Miller, J.L. Smith and G.A. Somkuti. New York: Elsevier.

Buchanan, R.L., 1988. Advances in cultural methods for the detection of *Listeria monocytogenes,* Society for Industrial Microbiology Comprehensive Conference on *Listeria monocytogenes,* Rohnert Park, California, Oct. 2-5, Abstract. I-14.

Ceylan, E., Fung, D.Y.C. and Sabah, J.R. 2004. Antimicrobial activity and synergistic effect of cinnamon with sodium benzoate or potassium sorbate in controlling *Escherichia coli* 0157:H7 in apple juice. *Journal of Food Science***,** 69:FMS102-FMS106.

Charpentier, E. and Courvalin, P. 1999. Antibiotic resistance in *Listeria* spp. *Antimicrobial Agents and Chemotherapy*, 43:2103-2108.

Cheroutre-Vialette, M., Lebert, I., Hebraud, M., Labadie, J.C. and Lebert, A. 1998. Effects of pH or a(w) stress on growth of *Listeria monocytogenes. International Journal of Food Microbiology*, 42:71-77.

Crozier-Dodson, E.A., Carter, M. and Zheng, Z. 2005. Process control: formulating food safety: an overview of antimicrobial ingredients. *Food Safety Magazine*. Dec 2004/Jan 2005.

Davies, J. 1998. Unanswered questions concerning antibiotic resistance. *Clinical Microbiology and Infection*, 4:2-3.

Davis, G.H.G., Fomin, L., Wilson, E. and Newton, K.G. 1969. Numerical taxonomy of *Listeria,* streptococci and possibly related bacteria. *Journal of General Microbiology*, 57:333-348.

Davis, M.J., Coote, P.J. and O'Byrne, C.P. 1996. Acid tolerance in *Listeria monocytogenes*: the adaptive acid tolerance response (ATR) and growth –phase-dependent acid resistance. *Microbiology,* 142:2975-2982.

De Luca, G., Zanetti, F., Fateh-Moghadm, P. and Stampi, S. 1998. Occurrence of *Listeria monocytogenes* in sewage sludge. *Zentralbl. Hyg. Umweltmed*. 201:269-277.

Dijkstra, R. 1987. Listeriosis in animals-clinical signs, diagnosis and treatment. In Listeriosis-joint WHO/ROI consultation on prevention and control, ed. A. Schonberg. West Berlin, December 10-12, 1986, pp. 68-76. Institut für Veterinärmed. des Bundesgesundheitsamtes, Berlin.

Doyle, M.E. 1999. Use of organic acids to control *Listeria* in meat. http://www.amif.org/ht/a/GctDocumentAction/i/7426. Accessed 26 February 2011.

Doyle, M.P. 1994. The emergence of new agents of foodborne diseases in the 1980s. *Food Research International,* 27:219-226.

Duche, O., Tremoulet, F., Namane, A. and Labadie, J. 2002. A proteomic analysis of the salt stress response of *Listeria monocytogenes*. *FEMS Microbiology Letters*, 215:183-188.

El Marrakchi, A., Hamama, A. and El Othmani, F. 1993. Occurrence of *Listeria monocytogenes* in milk and dairy products produced or imported into Morocco. *Journal of Food Protection*, 56:256-259.

Fenlon, D.R., Wilson, J. and Donachie, W. 1996. The incidence and level of *Listeria monocytogenes* contamination of food sources at primary production and initial processing. *Journal of Applied Bacteriology***,** 81:641-650.

FDA/USDA/CDC. 2003. Quantitative assessment of relative risk to public health from food borne *Listeria monocytogenes* among selected categories of ready-to-eat foods. (Online.) http://www.cfscan.fda.gov. Accessed 18 March 2009.

FDA/CDC, 2003. Reducing the risk of *Listeria monocytogenes*: FDA/CDC 2003 update of the Listeria Action Plan. (Online.) http://www.foodsafety.gov. Accessed November 2010.

Food and Agriculture Organization of the United Nations/World Health Organization Food Standards Programme. 2005. Proposed draft guidelines on the application of general principles of food hygiene to the [control] of *Listeria monocytogenes* in foods. Codex committee on food hygiene. In Codex Alimentarius. CX/FH 05/37/5.

Francis, G.A. and O'Beirne, D. 2006. Isolation and pulsed-field gel electrophoresis typing of *Listeria monocytogenes* from modified atmosphere package fresh-cut vegetables collected in Ireland. *Journal of Food Protection***,** 69:2524-2528.

Freesu, S.B. and Jones, D. 1988. Taxonomic studies on *Brochothrix, Erysipelothrix, Listeria* and atypical lactobacilli. *Journal of General Microbiology***,** 134:1165-1183.

Garrec, N., Picard-Bonnaud, F. and Pourcher, A.M. 2003. Occurrence of *Listeria* sp. and *L. monocytogenes* in sewage sludge used for land application: Effect of dewatering, liming and storage in tank on survival of *Listeria* species. FEMS *Immunology Medical Microbiology*, 35:275-283.

Gandhi, M. and Chikindas, M.L. 2006. *Listeria*: A food borne pathogen that knows how to survive. *International Journal of Food Microbiology*, 113:1–15.

Gilbert, R.J., McLauchlin , J. and Velani, S.K. 1993. The contamination of pate by *Listeria monocytogenes* in England and Wales in 1989 and 1990. *Epidemiology and Infection*, 110:543-551.

Harris, L.J., Farber, J.N., Beuchat, L.R., Parish, M.E., Suslow, T.V., Garret, E.H. 2003. Outbreaks associated with fresh produce: Incidence, growth and survival of pathogens in fresh and fresh-cut produce. *Comprehensive Reviews in Food Science and Food Safety*, Vol 2. Issue Supplement S1:78-141.

Hof, H. 1991. Therapeutic activities of antibiotics in listeriosis. *Infection*, 19**:**229-233.

Huang, N., Ho, C and McMillin, K.W. 2005. Retail shelf-life of pork dipped in organic acid before modified atmosphere or vacuum packaging. *Journal of Food Science,* 70:M382-M387.

Jacquet, C., Catimel, B., Brosch, R., Buchrieser, C., Dehaumont, P., Goulet, V., Lepoutre, A., Veit, P. and Rocourt, J.1995. Investigations related to the epidemic strain involved in the French listeriosis outbreak in 1992. *Applied and Environmental Microbiology*, 61:2242-2246.

Kimberling, C.V. 1988. Diseases of the central nervous system. In Jensen and Swift's diseases of sheep, 3rd ed. Philadelphia: Lea and Febiger, pp.195-199.

Kriem, M.R., El Marrakchi, A. and Hamarna, A. 1998. Prevalence of Listeria species on a variety of meat products in Morocco. Microbiology Aliments Nutrition, 16:179-187.

Kroll, R.G. and Patchett, R.A. 1992. Induced acid tolerance in *Listeria monocytogenes*. *Letters in Applied Microbiology*, 14:224-227.

Kruse, H. and Sorum, H. 1994. Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. *Applied and Environmental Microbiology,* 60:4015-4021.

Levy, S.B. 1998. The challenge of antibiotic resistance. Scientific American March 1998 ed. New York: Scientific American Inc.

Lou, Y.Q. and Yousef, A.E. 1997. Adaptation to sublethal environmental stresses protects *Listeria monocytogenes* against lethal preservation factors. *Applied Environmental Microbiology*, 63:1252-1255.

McEntire, J.C., Montville, T.J. and Chikindas, M.L. 2003. Synergy between nisin and select lactases against *Listeria monocytogenes* is due to the metal cations. *Journal of Food Protection,* 66:1631-1636.

McLauchlin, J. 1996. The relationship between *Listeria* and listeriosis. *Food Control,* 7:187-193.

Mead, P.S., Slutsker, L., Dietz, V., Mccaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M. and Tauxe, R.V. 1999. Food-related illness and death in the United States. *Emergency Infectious Diseases*, 5:607-625

.

Morvan, A., Moubareck, C., Leclercq, A., Hervé-Bazin, M., Bremont, S., Lecult, M., Courvalin, P. and Lê Monnier, A. 2010. Antimicrobial Résistance of *Listeria monocytogenes* Strains Isolated from Humans in France. *Antimicrobial Agents and Chemotherapy,* 54:2728-2731.

Norton, D.M. and Braden, C.R. 2007. Foodborne Listeriosis in E.T. Ryser and E.H. Marth (Ed.). *Listeria,* Listeriosis and Food Safety (3rd Ed.). CRC Press, Boca Raton, FL, pp 305-349.

Ottaviani, E., Ottaviani, M. and Agosti, M. 1997. Differential agar medium for *Listeria monocytogenes. Industrie Alimentari*, 36:888.

Phan-Thanh, L., Mahouin, F. and Aligè, S. 2000. Acid responses of *Listeria monocytogenes*. *International Journal of Food Microbiology*, 55:121-126.

Perreten, V., Schwarz, F. Cresta, L., Boeglin, M., Dasen, G. and Teuber, M. 1997. Antibiotic resistance spread in food. *Nature*, 389:801-802.

Rao, G. 1998. Risk factors for the spread of antibiotic-resistant bacteria. *Drugs*, 55:323-330.

Rocourt, J. and Cossart, P. 1997. *Listeria monocytogenes.* In Doyle, M.P., Bucchat, L.R. Montville, T.J. (Eds), Food Microbiology – Fundamentals and Frontiers. American society for Microbiology press. Washington DC. pp 337-352.

Rocourt, J. And Buchrieser, C. 2007. The genus *Listeria* and *Listeria monocytogenes:* Phylogenetic Position, Taxonomy and Identification in E.T. Ryser and E.H. Marth (Ed.). *Listeria,* Listeriosis and Food Safety (3rd Ed.). CRC Press, Boca Raton, FL, pp 1-20.

Raybaudi-Massilia, R.M., Mosqueda-Melger, J. and Martin-Belleso, O. 2009. Antimicrobial activity of malic acid against *Listeria monocytogenes, Salmonella*

enteritidis and *Esherichia coli* 0157:H7 in apple, pear and melon juices. *Food Control*, 20:105-112.

Sauders, B.D. and Wiedmann, M. 2007. Ecology of *Listeria* Species and *L. Monocytogenes* in the Natural Environment in E.T. Ryser and E.H. Marth (Ed.). *Listeria,* Listeriosis and Food Safety (3rd Ed.). CRC Press, Boca Raton, FL, pp 21-54.

Schlech, W.F., Lavigne, P.M., Bortolussi, R.A., Allen, A.C., Haldane, E.V., Wort, A.J., Hightower, A.W., Johnson, S.E., King, S.H., Nicholls, E.S. and Broome, C.V. 1983. Epidemic listeriosis-evidence for transmission by food. *New England Journal of Medicine,* 308:203-206.

Schlech, W.F. 2000. Epidemiology and clinical manifestations of *Listeria monocytogenes* infections. In Gram Positive Pathogens ed. Fischetti, V. et al. American Society for Microbiology Press, Washington DC, USA.

Sorrels, K.M., Enigl, D.C. and Harfield J.R. 1989. Effect of pH, acidulant, time and temperature on the growth and survival of *Listeria monocytogenes. Journal of Food Protection,* 52:571-573.

South African Department of Health 2001. Regulations governing microbiological standards for foodstuffs and related matters. Annexure A: Microbiological Methods, p 5.

http://www.doh.gov.za/docs/regulations/foodstuff/microbiological. Accessed 1 March 2010.

USDA/FSIS, U.S. Department of Agriculture/ Food Safety and Inspection Service, Background Document March 1992, Revised April 1992

Van Nierop, W., Dusé, A.G., Marais, E., Aithma, N., Thothobolo, N., Kassel, M., Stewart, R., Potgieter, A., Fernandes, J.S., Galpin, J.S. and Bloomfield, S.F. 2005. Contamination of chicken carcasses in Gauteng, South Africa, by *Salmonella, Listeria monocytogenes* and *Campylobacter*. *International Journal of Food Microbiology*, 99:1-6.

Van Renterghem, B., Huysman, F., Rygole, R. and Verstraete, W. 1991. Detection and prevalence of *Listeria monocytogenes* in the agricultural ecosystem. *Journal of Applied Bacteriology*, 71:211-217.

Vorster, S.M., Greebe, R.P. and Nortjè, G.L. 1993. The incidence of *Listeria* in processed meats in South Africa. *Journal of Food protection,* 56:169-172.

Walsh, D., Duffy, G., Sheridan, J.J., Blair, I.S. and McDowell, D.A. 2001. Antibiotic resistance among *Listeria,* including *Listeria monocytogenes,* in retail foods. *Journal of Applied Microbiology*, 90:517-522.

Watkins, J. and Sleath, K.P. 1981. Isolation and enumeration of *Listeria monocytogenes* from sewage, sewage sludge and river water. *Journal of Applied Bacteriology***,** 50:1-9.

Wesley, I.V. 2007. Listeriosis in Animals in E.T. Ryser and E.H. Marth (Ed.). *Listeria,* Listeriosis and Food Safety (3rd Ed.). CRC Press, Boca Raton, FL, pp 55-84.

Zagory, D. and Garren, D. 1999. HACCP: What It Is and What It Isn't. The Packer. August 16, 1999. http://www.nsf.org/business/nsf_agriculture/articles_haccp.pdf. Accessed 4 March 2011.

CHAPTER 2

ISOLATION AND IDENTIFICATION OF *LISTERIA* SPP. FROM VARIOUS FOOD PREMISES IN CENTRAL SOUTH AFRICA

2.1 INTRODUCTION

Isolation of *Listeria* from inoculated or naturally contaminated food and clinical specimens by use of nonselective media is a difficult process. Almost a century ago, Murray et al. (1926) stated that: "The isolation of the infecting organism is not easy and we found this to remain true even after we had established the cause of the disease". In recent years, various combinations of direct plating, cold enrichment, selective enrichment and several rapid methods are available to detect *L. monocytogenes* in clinical, food and environmental samples.

It is difficult to detect and isolate small numbers of *Listeria* from environmental and food samples that contain large numbers of indigenous microorganisms. In 1948, however, a young graduate student recognised the benefits of low-temperature incubation for recovering *L. monocytogenes* from clinical specimens. According to Gray et al. (1948), *L. monocytogenes* was only isolated in three of five bovine listeriosis cases after brain tissue was diluted in tryptose broth, stored for 5 to 13 weeks at 4 ˚C and then plated onto tryptose agar. This clearly demonstrated the ability of *L. monocytogenes* to multiply to detectable levels during extended storage at refrigeration temperature.

Gray's cold enrichment method was soon adopted as the standard procedure for recovering *L. monocytogenes.* Although this procedure is slow and labour intensive, it greatly enhances the isolation of *Listeria* (if present) from a variety of specimens, including food. In a study by Ryser et al. (1985) cottage cheese samples inoculated with

L. monocytogenes, yielded 43 of 112 (38.4%) samples after the samples were stored at 3˚C for up to 28 days, whereas cold enrichment of the same samples in tryptose broth for up to 8 weeks yielded *Listeria* in 50 of 112 (52.7%) samples.

The growth of *L. monocytogenes* is favoured at 4˚C but other organisms, including *Proteus, Pseudomonas, Hafnia,* enterococci and certain lactic acid bacteria are also able to multiply in non-selective media at refrigeration temperatures. This renders the detection of *Listeria* even more difficult (Albritton et al., 1980).

Availability of improved selective media and methods led investigators to the conclusion that cold enrichment offers no advantages over selective enrichment. The extended incubation period necessary for cold enrichments makes this procedure impractical for routine analysis of foods (Donnelly and Nyachuba, 2007). Cold enrichment has been a time consuming process and it was, therefore necessary to find a method with a shorter incubation period. In 1950 *L. monocytogenes* was isolated from an inoculation of nutrient broth containing 0.05% potassium tellurite. Contaminated material was inoculated into the nutrient broth and incubated at 37˚C for 6 to 8 h before being plated on tryptose agar with or without 0.05% potassium tellurite (Gray et al., 1950).

L. monocytogenes is resistant to various selective agents, including chemicals, antimicrobials and dyes. This resistance was therefore utilised to formulate a media that would enhance the growth of this pathogen and inhibit the growth of indigenous

bacterial flora. Selective agents that may be useful are included in Table 2.1 (Donnelly and Nyachuba, 2007):

Table 2.1: Potential selective reagents for enhancement of *Listeria* isolation

Selective agent	Effect
Potassium tellurite	*Listeria* reduces tellurite to tellurium, producing black colonies
Naladixic acid	Inhibitory to Gram-negative bacteria
Acriflavine	Inhibitory to Gram-positive cocci
Polymyxin B	Prevents growth of Gram-negative rods and streptococci
Moxalactam	Broad spectrum inhibitor to many Gram-positive and Gram-negative contaminants

2.1.1 Selective media for enrichment and isolation of *Listeria*

Fraser broth

Fraser broth is a modification of United States Department of Agriculture (USDA) LEB II medium consisting of proteose peptone (5.0 g/l), tryptone (5.0 g/l), Lab-Lemco powder (5.0 g/l), yeast extract (5.0 g/l), sodium chloride (20.0 g/l), disodium phosphate-2-

hydrate (12.0 g/l), potassium phosphate monobasic (1.35 g/l), naladixic acid (20 mg/l) and acriflavine HCl (25 mg/l), (McClain and Lee, 1987) and now also contains lithium chloride (3.0 g/l) and ferric ammonium citrate (0.5 g/l). In the presence of *Listeria* this broth has the advantage of turning black as a result esculin hydrolysis within 48 h of incubation. The USDA protocol has, therefore, replaced USDA LEB II medium with fraser broth as preferred secondary enrichment medium for meat, poultry and environmental samples (Johnson, 1998). Fraser broth is used as primary and secondary enrichment medium for the recovery of *Listeria.* However, no single selective enrichment broth has proven to be completely reliable for analysis of food products containing *Listeria* (Donnelly and Nyachuba, 2007).

Oxford Agar

In 1989, Curtis et al. developed Oxford agar, a medium prepared from Columbia agar base to which several selective agents were added. These selective agents include colistin sulfate (20 mg/l), fosfomycin (10 mg/l), cefotetan (2 mg/l), cycloheximide (400 mg/l), lithium chloride (15 g/l) and acriflavine (5 mg/l). Esculin and ferric ammonium citrate were added to produce black *Listeria* colonies from esculin hydrolysis.

Chromocult Listeria *selective agar*

The rich basis of Chromocult *Listeria* selective agar ensures rapid growth of a broad range of bacteria. Inhibitors are added to reduce growth of the majority of Gram-positive and Gram-negative pathogens, as well as yeasts and fungi. Addition of D-glucopyranoside enables visualisation of *Listeria* in the form of blue-green colonies

because of the production of D-glucosidase. *L. monocytogenes* also produces the enzyme phosphatidylinositol phospholipase C and the phospholipase activity can be seen in the formation of opaque haloes around *L. monocytogenes* colonies. L-phosphatidylinositol is added to the medium to detect these haloes. Colonies that appear blue-green with an opaque halo on the medium are, therefore, suspected to be *Listeria* (Ottaviani et al., 1997).

2.1.2 Rapid methods for detection of *Listeria*

Rapid test kits and systems are commercially available for the detection of *Listeria.* Many of these have received validation approval from the International Association of Analytical Communities (AOAC). Assay formats include latex bead-based lateral flow immunoassay, enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunofluorescence assay (ELFA), colorimetric DNA probe, immunomagnetic separation (IMS), fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR) based methods. Most methods require selective enrichment for up to 48 h (Elliot and Elmer, 2007).

2.1.3 Polymerase chain reaction (PCR)

PCR is the most widely reported rapid method for detection of *Listeria*, in particular *L. monocytogenes* (Levin, 2003) and this method has become more accessible to numerous specialist laboratories. Automated detection of PCR products, which exclude labour-intensive post-amplification, and the availability of commercial prepackaged reagents make it easier for laboratories to use this rapid method. However, PCR is still

not used in the food industry because of the high cost of instrumentation and a lack of standardised and validated methods for PCR detection of pathogens in foods (Malorny et al., 2003). A number of errors can also affect the specificity of the final test. Target sequences should be specific and primer specificity should be validated experimentally against a well-characterised reference strain panel that consist of both target and non-target organisms (Aznar and Alarcón, 2002). Various components in food can also interfere with the PCR reaction. Such PCR inhibitors include fats, proteins and primer-degrading nucleases. Certain components of selective media used to enrich for *Listeria* may also have inhibitory activity, including acriflavin, esculin, bile salts and ferric ammonium citrate (Scheu et al., 1998).

The aim of this study was to apply and evaluate various methods to isolate and identify *Listeria* spp. from various food production premises and RTE foodstuffs.

2.2 MATERIALS AND METHODS

2.2.1 Sampling methods

A total of 200 samples were collected from August 2009 to February 2010. Samples were collected from a red meat abattoir, a poultry abattoir, a dairy outlet and ready-to-eat (RTE) samples purchased from various outlets in the Bloemfontein and Kroonstad area.

Meat abattoir

Fifty red meat samples were collected and included 16 from different sites on pigs, 14 beef samples, 18 samples from sheep and two water samples from the drainage water of the abattoir. Samples were cut from the carcasses before final washing with a sterile butcher's knife, placed into sterile whirl packs and stored on ice in a cooler bag. Samples from sheep included faeces and intestines as well as swabs from the mouth and nostrils of the sheep. Samples were transported to the laboratory in a cooler bag immediately after collection and stored in a fridge until processing.

Poultry abattoir

Fifty samples were collected in two batches of 25 each, two weeks apart. Samples were taken at receiving and killing, de-feathering and evisceration. These included swabs from the skin of the poultry after de-feathering, different pieces of slaughtered chicken, water samples after rinsing of the whole chicken, water after de-feathering, brine water samples and different frozen portions. Samples were collected, transported, stored and analysed similar to previously reported.

Dairy samples

Fifty samples were collected from a dairy outlet in Bloemfontein. Samples were collected in two batches of 25 each over a two day period, one week apart. These included raw milk, pasteurised milk, cottage cheese, raw yoghurt, processed yoghurt, fruit juice, cheddar cheese and whey. Samples were collected aseptically from the

processing tanks into Falcon tubes, placed on ice in a cooler bag, transported to the laboratory and processed without delay.

Ready to eat samples (RTE)

Ready-to-eat samples were purchased from various outlets in Bloemfontein, the first 25 samples included a variety of food including sliced ham, salads, pasta dishes, mixed cold meat, ciabata filled with bacon and russians. The remaining 25 samples were different sushi samples collected from five different restaurants in the city. These samples included smoked salmon, crab and prawn.

2.2.2 Culturing procedures

Reference *Listeria* strains used as controls throughout the study included *Listeria monocytogenes* ATCC 19111, *Listeria welshimeri* ATCC 35897, *Listeria innocua* ATCC 33090, *Listeria seeligeri* ATCC 35967, *Listeria ivanovii* ATCC 19119 and *Listeria grayi* ATCC 25401, supplied by Quantum Biotechnologies (Randburg, RSA). The samples were diluted with 100 µl of brain heart infusion broth and inoculated onto brain heart infusion agar plates. After incubation at 35˚C for 24 h one loop-full of growth from agar plates was stored at −70˚C in Microbanks (Davies Diagnostics, Randburg, RSA).

Isolation was performed by using a slightly modified method of the EN ISO 11290-1/A1:2004 standard method (Figure 2.1). Dairy samples (25 ml) were pre-enriched by the addition of 225 ml half-Fraser broth. Pre-enrichment of all other food samples was

performed by placing 25 g of sample into a stomacher bag containing 225 ml half-Fraser broth dehydrated base (Bio-Rad, Johannesburg, RSA) and stomached for 2 min in a Seeward Stomacher 400 (Lab Systems, Victoria, Australia). A commercial supplement for half-Fraser broth was added to the base and samples were incubated at 35˚C for 24 h. After incubation 1 ml was collected for PCR pre-screening, 0.1 ml was inoculated into 10 ml full-Fraser broth for selective enrichment and one loopful was inoculated onto Oxford agar plates (Merck, Bellville, RSA) and incubated at 35˚C for 24 h. Fraser broth was incubated at 35˚C for 48 h after which one loop was inoculated onto *Listeria* agar (according to Ottaviani et al., 1997) (Merck) and another onto Oxford agar. Plates were again incubated at 35˚C for 24 – 48 h.

Colonies that appeared turquoise-blue with opaque halo colonies on *Listeria* agar and colonies that hydrolysed aesculin on Oxford agar were Gram-stained and checked for cellular motility. Gram-positive, catalase positive bacilli with tumbling motility at room temperature under microscope were confirmed as *Listeria* by API (Analytical Profile Index) *Listeria* (BioMèrieux, Randburg, RSA). Presumptive positive colonies were sub-cultured onto *Listeria* agar and incubated for 24 h at 35˚C. API test strips were inoculated with the pure culture according to the instructions of the manufacturer. After incubation, reagents were added to test strips and identification obtained via numerical profiling. *Listeria monocytogenes* ATCC 19111 was included as positive control and all positive isolates were stored in Microbanks at −70˚C.

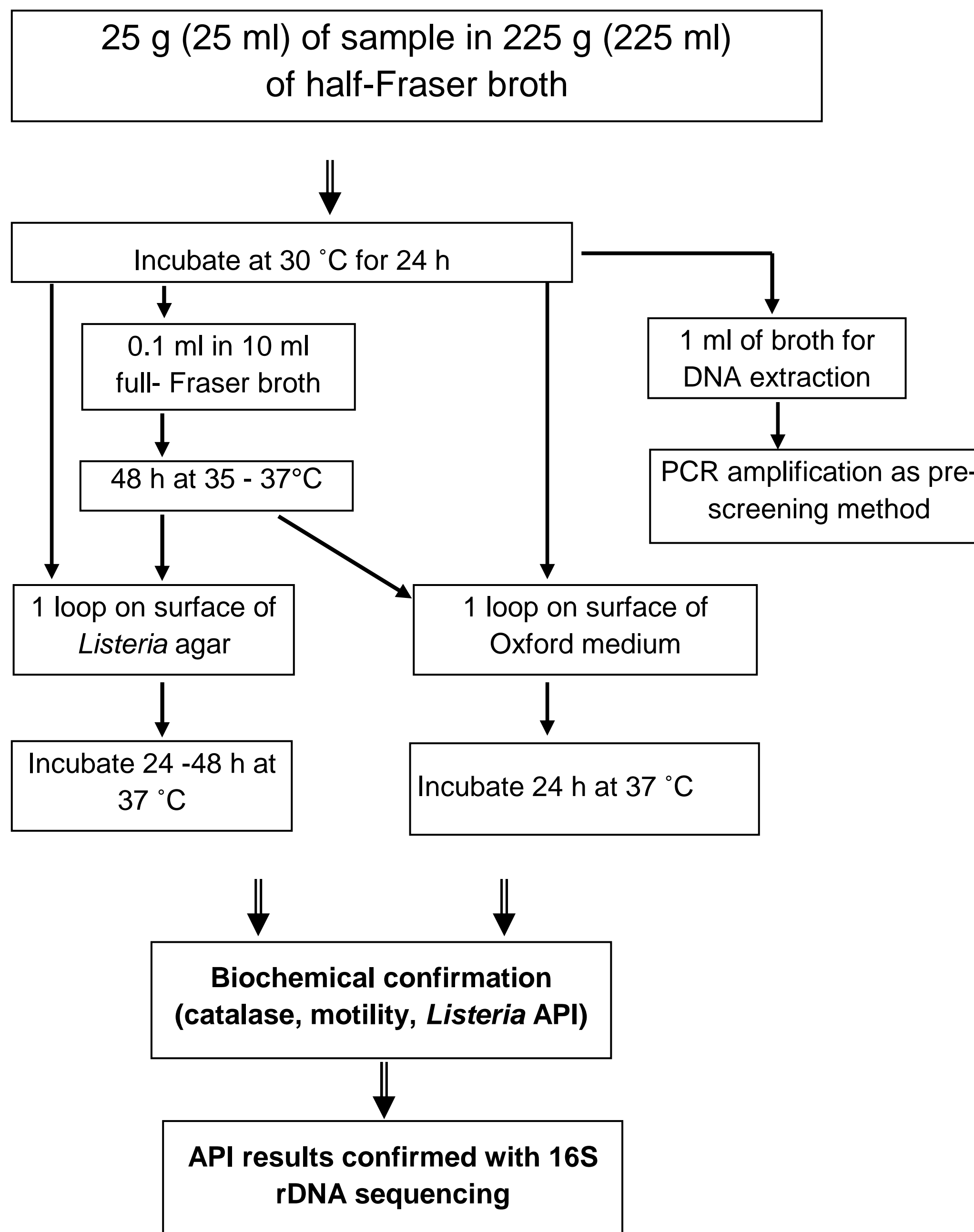

Figure 2.1 Diagrammatic representation of the method followed for detection of *Listeria spp.*

2.2.3 DNA based methodologies

Templates used for DNA based applications originated from the following: (a) the six mentioned *Listeria* control strains for testing the specificity of the primers (b) *L. monocytogenes, L. welshimeri* and *L. innocua* dilution series for determining PCR detection limits, and (c) API positive cultures for 16S rRNA sequencing.

2.2.3.1 Template preparation

(a) The six *Listeria* control strains included *L. monocytogenes, L. innocua, L. welshimeri, L. ivanovii, L. seeligeri and L. grayi* as previously described. Strains were inoculated on brain heart infusion agar plates. After incubation at 35˚C for 24 h adequate amounts of culture growth were suspended in 1 ml half-Fraser broth using an inoculation loop. Samples were centrifuged for 3 min at 3 000 rpm and DNA extracted from the pellets. Genomic DNA was extracted from the control strains using the PrepMan Ultra Sample Kit (Applied Biosystems, Halfway House, RSA), following the instructions as described by the manufacturer. One hundred µl of sample preparation reagent was aseptically added to each pellet, vortexed vigorously to resuspend the pellet and heated (100˚C) for 10 min. The tubes were then cooled to room temperature for 2 min and 50 µl of the supernatant was transferred to a new tube and stored at -20˚C. A 1:10 dilution was also prepared for each DNA sample and stored under the same conditions.

(b) *L. monocytogenes, L. welshimeri* and *L. innocua*. controls were inoculated from the Microbanks onto brain heart infusion agar and incubated at 35˚C for 24 h. A

suspension with an OD $_{600\ nm}$ absorbance value of 0.2 was prepared for each strain in half-Fraser broth, as well as a ten fold serial dilution ranging from 10^{-1} to 10^{-10}. A volume of 100 µl from each dilution was spread onto brain heart infusion agar and incubated for 48 h at 35˚C. This was performed in duplicate and the remainder of the dilution kept in the fridge. After incubation growth was enumerated and according to the CFU.ml^{-1} values obtained, further dilutions of 100 CFU.ml^{-1}, 50 CFU.ml^{-1}, 10 CFU.ml^{-1} and 5 CFU.ml^{-1} were prepared from the suitable originals. Two aliquots (1 ml each) from each dilution were centrifuged for 5 min at 3 000 rpm. The pellet from one aliquot was subjected to DNA extraction using the PrepMan Ultra Sample Kit (Applied Biosystems) and the second was resuspended in 100 µl half-Fraser broth and subjected to lysis by boiling for 10 min. Samples were cooled on ice for 2 min and centrifuged at 13 000 rpm for 5 min at 4˚C. The supernatants were kept on ice and used directly as templates for PCR analysis.

(c) Pure growth from the isolates identified as *Listeria* with API was suspended in 1 ml sterile water and centrifuged for 3 min at 3 000 rpm at room temperature. Pellets were subjected to DNA extraction using a harsh lysis method (Labuschagne and Albertyn, 2007). Lysis buffer (50 µl) and 0.2 ml glass beads were added to each pellet. Samples were vortexed for 2 min and kept on ice after which 7 M ammonium acetate (275 µl) was added to each tube. The tubes were incubated at 65˚C for 5 min, followed by incubation on ice for 5 min. Chloroform (500 µl) was added to the supernatant and the suspension vortexed for 3 sec. Samples were centrifuged at 13 000 rpm for 2 min at 25˚C and the supernatants transferred to

new tubes. An equal volume of isopropanol was added and precipitation allowed for 5 min at 25˚C. Samples were centrifuged at 13 500 rpm for 2 min at 4˚C, washed once with 250 µl ice cold 70% ethanol and the pellets dried in the CentriVac™ DNA concentrator (Labconco, Johannesburg, RSA) for 5–10 min. The pellets were resuspended in 50 µl TE buffer (10 mM Tris, 1 mM EDTA) with RNase and incubated at 37˚C for one hour. DNA was stored at −20˚C until further use.

2.2.3.2 PCR set up and conditions

(a) The primer pair List-univ-F (5'-ATGTCATGGAATAA-3') and List-univ-R (5'-GCTTTTCCAAGGTGTTTTT-3') (Cocolin et al., 2002) targeting the *iap* gene encoding the invasion-associated protein p60 in all *Listeria* spp. were used (Bubert et al., 1992). PCR was performed in a final volume of 25 µl in the G-Storm GS482 thermal cycler (Gene Technologies, Pretoria, RSA). The reaction mixture consisted of 5 µl extracted DNA, 1X reaction buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 0.2 mM dNTPs, 0.5 µM (each) primers and 1.25 U of Super-Therm DNA polymerase (Southern Cross Biotechnology, Cape Town, RSA). The reaction conditions were 95˚C for 2 min, followed by 35 cycles of 95˚C for 1 min, 36˚C for 2 min, 72˚C for 3 min and a final cycle of 72˚C for 7 min. The products were analysed by electrophoresis on a 2% agarose gel stained with Gold View (0.05%) and visualised under UV light. To determine the optimum annealing temperature a gradient PCR was performed. The reaction conditions were 92˚C for 5 min, followed by 35 cycles of annealing at 95˚C for 1 min, temperatures ranging from 36˚C to 50˚C for 1 min, and 72˚C for 3 min. A final elongation step at 72˚C for

7 min was also included. Seeing that the gradient PCR still yielded non-specific binding, a touch down PCR was performed in an attempt to increase the sensitivity and specificity of the amplification. The touch down PCR consisted of one cycle at 95˚C for 3 min, followed by 10 cycles of 95˚C for 30 sec, 47˚C for 30 sec and 72˚C for 45 sec. A further 25 cycles of 95˚C for 30 sec, 36˚C for 30 sec and 72˚C for 45 sec were included with a final elongation cycle at 72˚C for 7 min.

(b) Two PCR amplifications were performed on the three control strains, *L. monocytogenes, L. innocua* and *L. welshimeri,* using the *Listeria* universal primers as described in 2.2.3.2 (a) above and another pair amplifying the 16S rRNA gene region identified as 63-F (5'-CAGGCCTAACACATGCAAGTC-3') and -1387-R (5'-GGGCGGGTCACAAGGC-3') (Marchesi et al., 1998). The reaction mixture consisted of 5 µl DNA, 1X reaction buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 0.2 mM dNTPs, 0.5 µM (each) primers and 1.25 U of Super-Therm DNA polymerase in a final volume of 25 µl. The cycling parameters for the *Listeria* universal primers were the touch down PCR conditions in 2.2.3.2 (a) and the parameters for amplifying the 16S rRNA gene region were 94˚C for 3 min, followed by 30 cycles of 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 90 min and a final elongation cycle of 72˚C for 10 min.

(c) The same primer pair described in 2.2.3.2 (b) was used to amplify the 16S rRNA gene region of partially identified *Listeria* specimens isolated from food samples. PCR amplification was performed using the same reaction setup, reaction

conditions and thermal cycler as described in 2.2.3.2 (b) with the exception of DNA template volume which was in this case only 0.5 µl. The amplified products were analysed by electrophoresis on a 1% agarose gel, stained with Gold View (0.05%) and visualised under UV light.

2.2.3.3 DNA sequencing

16S rDNA PCR products generated in 2.2.3.2 (c) for each of the partially identified *Listeria* isolates, were subjected to DNA sequencing using primer 63-F. The reaction mixture consisted of 0.5 µl of previously amplified PCR product as template, buffer 1X (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 3.2 µM primer, and 0.5 µl premix (Big Dye® Terminator V3.1 Cycle Sequencing Kit, Applied Biosystems). The reactions were performed in a total volume of 10 µl using the G-Storm GS482 thermal cycler (Gene Technologies) and the cycling parameters were 96˚C for 1 min, followed by 25 cycles of 96˚C for 10 sec, 50˚C for 5 sec and 60˚C for 4 min. Post-reaction cleanup was performed using EDTA-ethanol method precipitation. EDTA (125 mM) and 60 µl absolute ethanol were added to the sequence products. After vortexing for 5 sec, DNA was precipitated at room temperature for 15 min. Samples were centrifuged at 4˚C for 15 min at 13 000 rpm and the pellet washed with 200 µl 70% ethanol. Pellets were dried in the Centrivac™ DNA concentrator (Labconco) for 5 min and stored at 4˚C. Sequencing was performed on an ABI Prism 3130 XL genetic analyser. The Basic Logal Alignment Search Tool (BLAST) was used to compare the obtained sequences to the National Center for Biotechnology Information (NCBI) GenBank database for identification.

2.3 RESULTS AND DISCUSSION

2.3.1 Isolation

The majority of the *Listeria* strains were isolated from poultry samples. None of the samples from the red meat abattoir were found to be positive for *Listeria,* whereas 80% (40/50) of the samples from the poultry abattoir were positive for *Listeria* spp. *Listeria monocytogenes* were cultured from two samples from the poultry abattoir, one sample was collected from the running water after the first rinsing of the carcasses before de-feathering and the other sample collected from the offal of the carcass.

The majority of *Listeria* strains had acceptable *Listeria* genus API profiles (Table 2.2). The *Listeria welshimeri/L. innocua* API profile (sample no 60) turned out to be *L. monocytogenes* when confirmed with 16S rRNA sequencing profiles, while the two *L. grayi* strains were identified as *L. innocua.* Table 2.3 represents a detailed list of the different species cultured from the 200 samples where the API profiles are compared with the 16S results. Six *Listeria* spp. were isolated from the RTE samples and *L. monocytogenes* was identified in four (16%) of the sushi samples. The dairy samples yielded six *Listeria* spp., but did not include any *L. monocytogenes.*

Table 2.2 API profiles of presumptive *Listeria* strains isolated from food and diary samples (n=52).

Samples	Profile/Description*	Strain
Chicken (38 samples)	7511/7711 acceptable to genus	*L. welshimeri*
Chicken (2 samples)	7510/7110 acceptable to genus	*L. innocua*
RTE food (2 samples)	7711 acceptable to genus	*L. welshimeri*
RTE food (2 sample)	7110/7510 acceptable to genus	*L. innocua*
RTE food (1 sample)	6550 acceptable to genus	*L. monocytogenes*
RTE food (1 sample)	7710	*L. welshimeri /L. innocua*
Dairy (2 samples)	7530	*L. grayi*
Dairy (4 samples)	7510 acceptable to genus	*L. innocua*

* Acceptable to genus when ID >98%

Table 2.3 *Listeria* isolates identified using API and 16S rDNA sequencing

Lab. No.	Origin	API	16S rDNA PCR
51 - 100	**Poultry abattoir**		
51	Piece found on floor	*L. welshimeri*	*L. welshimeri*
53	Water after de-feathering	*L. welshimeri*	*L. welshimeri*
54	Brine water	*L. welshimeri*	*L. welshimeri*
55	Drumstick	*L. welshimeri*	*L. welshimeri*
56	Wing	*L. welshimeri*	*L. welshimeri*
57	Small fillet	*L. welshimeri*	*L. welshimeri*
58	Large fillet	*L. welshimeri*	*L. welshimeri*
59	Bone after deboning	*L. welshimeri*	*L. welshimeri*
60	Offal (neck, intestines)	*L. welshimeri*	*L. monocytogenes*
61	Frozen piece	*L. welshimeri*	*L. welshimeri*
62	Ice from frozen pieces	*L. welshimeri*	*L. welshimeri*
63	Frozen piece	*L. welshimeri*	*L. welshimeri*
64	Ice scraping	*L. welshimeri*	*L. welshimeri*
65	Whole carcass	*L. welshimeri*	*L. welshimeri*
66	Stomach	*L. welshimeri*	*L. welshimeri*
67	Intestines	*L. welshimeri*	*L. welshimeri*
75	Feathers	*L. welshimeri*	*L. welshimeri*
76	Drain sample	*L. welshimeri*	*L. welshimeri*
77	Water after 1st rinse	*L. innocua*	*L. monocytogenes*
78	Water after de-feathering	*L. welshimeri*	*Bacillus* species
79	Water after de-feathering	*L. welshimeri*	*L. welshimeri*
80	Brine water	*L. welshimeri*	*L. welshimeri*
81	Drain sample	*L. welshimeri*	*L. welshimeri*
82	Feathers	*L. welshimeri*	*L. welshimeri*
83	Intestines	*L. welshimeri*	*L. welshimeri*
84	Head and claws	*L. innocua*	*L. innocua*
85	Intestines	*L. welshimeri*	*L. welshimeri*
86	Wing	*L. welshimeri*	*L. welshimeri*
87	Liver	*L. welshimeri*	*L. welshimeri*
88	Stomach	*L. welshimeri*	*L. welshimeri*
89	Stomach	*L. welshimeri*	*L. welshimeri*
91	Neck	*L. welshimeri*	*L. welshimeri*
92	Drumstick after brining	*L. welshimeri*	*L. welshimeri*
93	Fillet after brining	*L. welshimeri*	*L. welshimeri*
94	Fresh piece pre-packed	Mixed culture	*L. welshimeri*
95	Fresh piece pre-packed	*L. welshimeri*	*L. welshimeri*
96	Bone after deboning	*L. welshimeri*	*L. welshimeri*
97	Fillet	*L. welshimeri*	*L. welshimeri*
98	Fillet	*L. welshimeri*	*L. welshimeri*
100	Ice scrapings	*L. welshimeri*	*L. welshimeri*

Table 2.3 Continued

Lab. No.	Origin	API	16S rDNA PCR
101 – 150	**RTE samples**		
105	Tuna pasta	*L. innocua*	*L. innocua*
115	Pasta salad	*L. welshimeri*	*L. welshimeri*
126	Sashimi	*L. monocytogenes*	*L. monocytogenes*
138	California roll (prawn)	*L. welshimeri*	*L. monocytogenes*
139	Maki	*L. innocua*	*L. monocytogenes*
141	California roll (salmon)	*L. welshimeri/* *L. innocua*	*L. monocytogenes*
151 – 200	**Dairy samples**		
151	Raw milk	*L. grayi*	*L. innocua*
152	Raw milk	*L. innocua*	*L. innocua*
153	Raw milk	*L. grayi*	*L. innocua*
157	Raw yoghurt	*L. innocua*	*L. innocua*
158	Raw yoghurt	*L. innocua*	*L. innocua*
172	Raw milk	*L. innocua*	*L. innocua*

Milk and dairy products, various meats and meat products such as beef, pork, fermented sausages, fresh produce such as radishes, cabbage, seafood and fresh products have all been associated with *Listeria* contamination (Gandhi and Chikindas, 2007). *L. monocytogenes* has been found in a wide variety of raw and processed foods. Although *L. monocytogenes* is a major public health concern, the non-pathogenic species *L. innocua* and *L. welshimeri* are considered of interest in food microbiology since its presence indicates the potential presence of *L. monocytogenes* (Pellicer et al., 2004). Academia, government agencies and the food industry have aimed at developing new and improved methods to prevent the survival and growth of

Listeria. Ongoing efforts are in place to control the food-borne pathogen *Listeria* in foods and in food processing facilities. These efforts include improved monitoring and reporting of food-borne diseases by government agencies, routine food sampling and testing, inspection at food processing facilities, training of food workers, establishment of HACCP and general awareness among consumers about food safety (Bryan, 2002).

Currently, the FDA has a zero-tolerance policy in place for *L. monocytogenes* in ready-to-eat foods. Based on this policy, if any indication of *L. monocytogenes* is found in food, the product is regarded as adulterated. The FDA is reviewing a petition (2007) made by trade associations to change the zero-tolerance policy for *L. monocytogenes* in foods that do not support the growth of the organism. The petition has requested the establishment of a regulatory limit for *L. monocytogenes* of 100 CFU.g^{-1} in foods that do not support the growth of the microorganism (Todd, 2007). Based on the risk assessment published by the FDA and FSIS and other research reports (Chen et al., 2003), the petition proposes that concentrating on the number of *L. monocytogenes* present in a food rather than just its presence alone may be more effective in improving food safety and promoting public health (Gandhi and Chikindas, 2007). Tests for the detection of *Listeria* are being done routinely in the food industry. However, according to the Regulations Governing Microbiological Standards For Foodstuffs and Related Matters in South Africa no provision has been made for microbiological specifications with regards to *Listeria monocytogenes* (South African Department of Health, 2001).

2.3.2 DNA based results

L. monocytogenes ATCC 19111, *L. innocua* ATCC 33090, *L. welshimeri* ATCC 35897, *L. ivanovii* ATCC 19119, *L. seeligeri* ATCC 35967 and *L. grayi* ATCC 25401 were used as positive controls in attempting to establish a pre-screening PCR method for the detection of *Listeria* species directly from food samples, subject to 24 h enrichment. Genomic DNA extracted from each of the controls was used as template to amplify the *iap* gene using primer pair List-univ-F and List-univ-R (Cocolin et al., 2002). Each species should be, at least partially, identifiable by the length of the amplified product: 457 bp (*L. monocytogenes*), 472 bp (*L. innocua*), 610 bp (*L. welshimeri*), 610 bp (*L. ivanovii*), 601 bp (*L. seeligeri*) and unknown (*L. grayi*). Initially non specific binding was evident (Fig. 2.2), but PCR optimisation resulted in the successful amplification of the target gene in all but *L. grayi* (Fig. 2.3). Detection limits were determined using a dilution series of pure *L. monocytogenes, L. welshimeri* and *L. innocua* cultures representing 5–100 CFU.ml^{-1}. Genomic DNA was extracted from 1 ml of each dilution (using two different extraction methods), and used as template for amplification of the *iap* gene. The 16S rDNA gene was also targeted as a control, but no amplification was obtained for either of the targeted genes.

In the current study the possibility of using a PCR pre-screening method was considered for the early detection of *Listeria* species from 24 h enriched samples. Culturing techniques used to detect *Listeria* are laborious and time consuming and a pre-screening for the presence of *Listeria* might lighten the sample load. Although many studies have considered the use of PCR methods for *Listeria* detection the approach

usually implied numerous enrichment steps and some require considerable skill and financial means (Brehm-Stecher and Johnson, 2007). The *iap* gene targeted in this study has been demonstrated a reliable PCR target for differentiation of cultivated *Listeria* spp. (Bubert et al., 1992, Bubert et al., 1999 and Cocolin et al., 2002) and the applicability for its use on actual food samples seemed promising. The DNA extraction methods used were not laborious and required no specialised equipment. PCR reaction conditions had to be optimised to increase the sensitivity and specificity of the amplification as is often the case with inter laboratory duplication. No amplicon could be obtained for *L. grayi,* but since this species was not included in the study by Cocolin et. al. (2002) and it has not been detected in, or isolated from food samples to date, it was disregarded for the purpose of the present study.

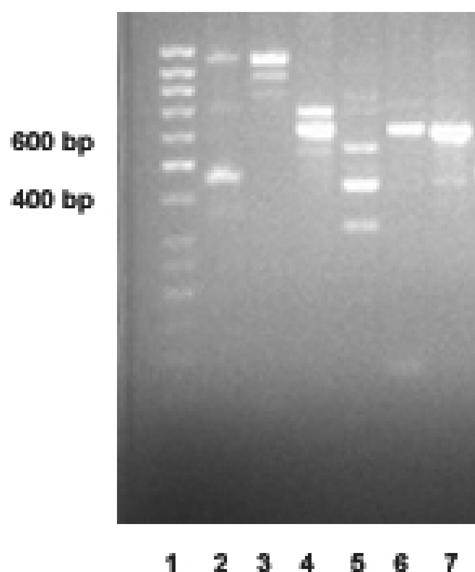

Fig. 2.2 **Agarose gel electrophoresis of *iap* gene products amplified from control *Listeria* genomic DNA. Lane 1 represents a 50bp DNA ladder (Fermentas); lane 2, *L. monocytogenes*; lane 3, *L. grayi;* lane 4, *L. welshimeri;* lane 5, *L. innocua;* lane 6, *L. ivanovii* and lane 7, *L. seelegeri.***

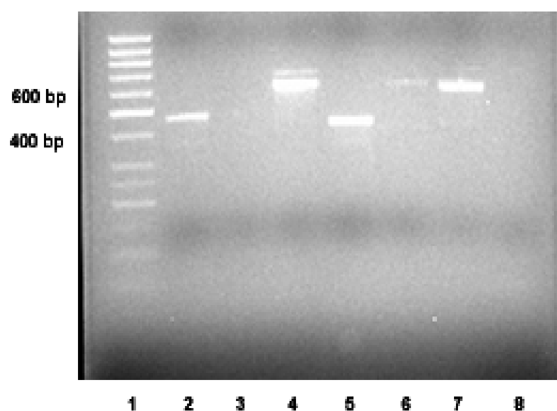

Fig. 2.3 **Agarose gel electrophoresis of *iap* gene products amplified from control *Listeria* genomic DNA after PCR optimisation. Lane 1 represents a 50bp DNA ladder (Fermentas); lane 2, *L. monocytogenes* (457 bp); lane 3, *L. grayi*; lane 4, *L. welshimeri* (610 bp); lane 5, *L. innocua* (472 bp); lane 6, *L. ivanovii* (610 bp) and lane 7, *L. seeligeri* (601 bp).**

To determine detection limits dilutions ranging from 5–100 CFU.ml^{-1} of the control strains were included. Quantitative assays based on the detection of virulence genes in *L. monocytogenes* have been tested on a variety of foods, and sensitivities reported to range from 1000 CFU.ml^{-1} (Bhagwat, 2003) to 1-5 CFU.25 ml^{-1} (or 1-5 CFU.25 g^{-1}) (Somer and Kashi, 2003). Countries including the United States, Australia and New Zealand applied a zero-tolerance policy for *L. monocytogenes* in foods (absent in 25 g food). The European legislation requires absence of *L. monocytogenes* in RTE food for certain consumer groups and allows limits of 100 CFU.g^{-1} in other categories of RTE foods (Food Safety Authority of Ireland, 2005).

Table 2.4 summarises some examples of detection limits determined for *L. monocytogenes* in food samples, using different PCR methods (Scheu, 1998).

Table 2.4: *Listeria monocytogenes* detection limits in various food products.

SAMPLE	GENE REGION	DETECTION LIMIT	REFERENCE
Milk	*hylA* promoter region	1 CFU/.300 ml^{-1}	Starbuck et al., 1992
Cheese, poultry	Cell surface protein associated gene	4-10 CFU/PCR	Wang et al., 1992a
Chicken, pork, beef	16S rRNA	2 X 10^{4} CFU.ml^{-1}	Wang et al., 1992b
Soft cheese	*Dth* 18 gene	2 X 10^{3}-2 X 10^{8} CFU.g^{-1}	Wemars et al., 1991a

No amplification of the *iap* gene could be detected, even above a concentration of 100 CFU.ml^{-1}. The 16S rDNA gene, included as control, also failed to amplify, which says more about the DNA extraction method that the gene chosen for screening. Although the extraction methods are highly recommended as being rapid and easy to perform with no specialised equipment needed, the applicability might rather be aimed at extracting DNA from excess target material, rather than the detection of limited amounts. Furthermore, the extraction methods did not allow for the detection of 100 CFU.ml^{-1} originating from a pure culture, which would not even consider the PCR inhibitors that may be present in food samples (for example fat, salts or acids). In conclusion, the results do not reflect on the applicability of the targeted *iap* gene in this case, since difficulty with the extraction method hampered any further downstream applications.

2.4 CONCLUSIONS

The presence of *Listeria* in food samples in the current study, is an indication of the importance of routine laboratory tests for the detection of *Listeria.* Although none of the *Listeria* spp. found in this study have been implicated in food-borne outbreaks, it is important to recognise their presence. Many of the food-borne diseases that result in diarrhea are usually not severe. However, more serious and prolonged illness can occur due to pathogens such as *L. monocytogenes* and usually requires antibiotic treatment (Gandhi and Chikindas, 2007). Approximately five people in South Africa die

each day from something they have ingested, while people suffering with AIDS, diabetes, heart disease or TB are highly susceptible and most at risk. However, food-borne diseases caused by *L. monocytogenes, Vibrio parahemoliticus* and *E. coli* 0157H:7, have unusually high mortality rates even among healthy individuals. Due to a lack of information, most of these deaths in South Africa go without notice, except to friends and family (handwashingforlife® South Africa, 2003). It can, therefore, be concluded that routine testing for *Listeria monocytogenes* is of utmost importance in both the clinical setup as well as the food production industry.

2.5 REFERENCES

Albritton, W.L., Williams, G.L., DeWitt, W.E. and Feeley, J.C. 1980. *Listeria monocytogenes.* In Manual of clinical microbiology, 3rd ed., eds. E.H. Lennette, A. Balows, W.J. Hausler, Jr. and J.P. Truant. Wshington, D.C.: American Society for Microbiology, pp.139-142.

Aznar, R. and Alarcón, B. 2002. On the specificity of PCR detection of *Listeria monocytogenes* in food: a comparison of published primers. *System Of Applied Microbiology*, 25:109-119.

Bhagwat, A. 2003. Simultaneous detection of *Escherichia coli* 0157:H7, *Listeria monocytogenes* and *Salmonella* strains by real-time PCR. *International Journal of Food Microbiology,* 84:217-224.

Brehm-Stecher, B.F. and Johnson, E.A. 2007. Rapid Methods for Detection of *Listeria* in E.T. Ryser and E.H. Marth (Ed.). *Listeria,* Listeriosis and Food Safety (3rd Ed.). CRC Press, Boca Raton, FL, pp 257-275.

Bryan, F.I. 2002. Where we are in retail food safety, how we got to, where we are, and how do we get there? *Journal of Environmental Health***,** 65:29-36.

Bubert, A., Hein, I., Rauch, M., Lehner, A., Yoon, B., Goebel, W. and Wagner, M. 1999. Detection and differentiation of *Listeria* spp. by a single reaction based on multiplex PCR. *Applied and Environmental Microbiology*, 65:4688-4692.

Bubert, A., Kohler, S. and Goebel, W. 1992. The homologous and heterologous regions within the *iap* gene allow genus-specific and species-specific identification of *Listeria* spp. by polymerase chain reaction. *Applied and Environmental Microbiology*, 58:2625-2632.

Chen, Y., Ross, W.H., Scott, V.N., and Gombas, D.E. 2003. *Listeria monocytogenes:* low levels equal low risk. *Journal of Food Protection,* 66:570-577.

Cocolin, L., Rantsiou, K., Lacumin, L., Cantoni, C. and Comi, G. 2002. Direct identification in food samples of *Listeria* spp. and *Listeria monocytogenes* by molecular methods. *Applied and Environmental Microbiology*, 68:6273-6282.

Curtis, G.D.W., Mitchell, R.G., King, A.F. and Griffen, E.J. 1989. A selective differential medium for the isolation of *Listeria monocytogenes. Letters of Applied Microbiology*, 8:95-98.

Donelly, C.W. and Nyachuba, D.G. 2007. Conventional Methods to Detect and Isolate *Listeria monocytogen* in E.T. Ryser and E.H. Marth (Ed.). *Listeria,* Listeriosis and Food Safety (3rd Ed.). CRC Press, Boca Raton, FL, pp 215-256.

Food Safety Authority of Ireland. 2005. The control and management of *Listeria monocytogenes* contamination of food. Available from http://www.fsai.ie. Accessed 5 September 2010.

Gandhi, M and Chikindas, M.L. 2007. *Listeria* : A foodborne pathogen that knows how to survive, *International Journal of Food Microbiology,* 113:1-15.

Gray, M.L., Stafseth, H.J., Thorp, F. Jr., Sholl, L.B. and Riley, W.F. Jr. 1948. A new technique for isolating listerellae from the bovine brain. *Journal of Bacteriology,* 55:471-476.

Gray, M.L., Stafseth, H.J. and Thorp, F. Jr. 1950. The use of potassium tellurite, sodium azide, and acetic acid in a selective medium for the isolation of *Listeria monocytogenes. Journal of Bacteriology***,** 59:443-444.

Handwashingforlife. 2003. Understanding Risk/ The 5 faces of Foodborne illness. (Online) http://za.handwashingforlife.com. Accessed 28 January 2011.

Johnson, J.L. 1998. Isolation and identification of *Listeria monocytogenes* from meat, poultry and egg products. In USDA-FSIS microbiology laboratory guidebook. 3rd ed., vol. 1.

Labuschagne, M. and Albertyn, J. 2007. Cloning of an epoxide hydrolase-encoding gene from *Rhodotorula mucilaginosa* and functional expression in *Yarrowia lipolytica*. *Yeast,* 24:69-78.

Levin, , R.E. 2003. Application of the polymerase chain reaction for detection of *Listeria monocytogenes* in foods: a review of methodology. *Food Biotechnology***,** 17:99-1161.

Malorny, B., Tassios, P.T., Rådström, P., Cook, N., Wagner, M. and Hoorfar, J. 2003. Standardization of diagnostic PCR for the detection of foodborne pathogens. *International Journal of Food Microbiology***,** 83:39-48.

Marchesi, J.R., Sato, T., Weightman, A.J., Martin, T.A., Fry, J.C., Hiom, S.J. and Wade, W.G. 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Applied and Environmental Microbiology,* 64:795-799.

McClain, D. and Lee, W.H. 1987. A method to recover *Listeria monocytogenes* from meats. Proc. Annual Meeting, American Society of Microbiology, Atlanta, May 1-6, Abstract P23.

Murray, E.G.D., Webb, R.A. and Swann, M.B.R. 1926. A disease of rabbits characterized by a large mononuclear leucocytosis, caused by a hitherto undescribed

bacillus, *Bacterium monocytogenes* (n. sp.). *Journal of Pathology Bacteriology*, 29:407-439.

Ottaviani, E., Ottaviani, M. and Agosti, M. 1997. Differential agar medium for *Listeria monocytogones. Industrie Alimentari*, 36:888.

Pellicer, K.E., Copes, J.A., Nosetto, E.O. and Echeverria, M.G. 2004. Characterisation of *Listeria* spp. isolated from ready-to-eat products in Argentina using SDS-PAGE and restriction endonuclease. *Food Research International,* 37:1013-1019.

Ryser, E.T., Marth, H. and Doyle, M.P. 1985. Survival of *Listeria monocytogenes* during manufacture and storage of cottage cheese. *Journal of Food Protection*, 50:7-13.

Scheu, P.M., Berghof, K. and Stahl, U. 1998. Detection of pathogenic and spoilage micro-organisms in food with the polymerase chain reaction. *Food Microbiology*, 15:13-31.

Somer, L. and Kashi, Y. 2003. A PCR method based on 16S rRNA sequence for simultaneous detection of the genus *Listeria* and the species *Listeria monocytogenes* in food products. *Journal of Food Protection*, 66:1658-1665.

South African Department of Health 2001. Regulations governing microbiological standards for foodstuffs and related matters. Annexure A: Microbiological Methods, p 5.

http://www.doh.gov.za/docs/regulations/foodstuff/microbiological. Accessed 1 March 2010.

Starbuck, M.A.B., Hill, P.J. and Stewart, G.S.A.B. 1992. Ultra sensitive detection of *Listeria monocytogenes* in milk by the polymerase chain reaction (PCR). *Letters of Applied Microbiology***,** 15:248-252.

Todd, E.C.D. 2007. *Listeria:* Risk Assessment, Regulatory Control and Economic Impact in E.T. Ryser and E.H. Marth (Ed.). *Listeria,* Listeriosis and Food Safety (3rd Ed.). CRC Press, Boca Raton, FL, pp 767-812.

Wang, R.F., Cao, W.W. and Johnson, M.G. 1992a. Development of cell surface protein associated gene probe specific for *Listeria monocytogenes* and detection of the bacteria in food by PCR. *Molecular Cellular Probes***,** 6:119-129.

Wang, R.F., Cao, W.W. and Johnson, M.G. 1992b. 16S rRNA-based probes and polymerase chain reaction method to detect *Listeria monocytogenes* cells added to foods. *Applied Environmental Microbiology***,** 58:2827-2831.

Wernars, K., Heuvelman, C.J., Ghakaraborty, T. and Notermans, SH.W. 1991a. Use of the polymerase chain reaction for direct detection of *Listeria monocytogenes* in soft cheese. *Journal of Applied Bacteriology***,** 70:121-126.

CHAPTER 3

COMPARING ANTIBIOTIC AND ORGANIC ACID SUSCEPTIBILITY IN *LISTERIA* SPP.

3.1 INTRODUCTION

Antibiotics have for many years been used to control bacterial diseases in livestock and also as growth promoters. However, this practice has been reported to result in the development of resistance to one or more antibiotics in food-borne pathogens, as bacteria are capable of adapting to unfavourable environmental conditions. When this organism is exposed to sub-lethal levels of an antimicrobial agent, it can adapt and develop resistance to higher levels of the antimicrobial as well as cross-resistance to other agents (Gandhi and Chikindas, 2007). Because bacteria have the ability to develop resistance to every antibiotic, it is anticipated that bacterial species which are still considered susceptible to most antibiotics will become resistant (Faleiro et al., 2003). In recent years reports have increased on emerging antimicrobial resistance in the food-borne pathogen *Listeria* (Gandi and Chikindas, 2007). This organism has until recently been regarded as highly susceptible. *L. monocytogenes,* for example, is frequently found in the digestive tract in humans and animals where various *Enterococcus* and *Streptococcus* are prevalent in high numbers and which can harbour resistance genes, contributing to the problem (Faleiro et al., 2003).

Because of their natural origin and activity as antimicrobial agent, antioxidant, flavouring and acidifying agents as well as their low cost, organic acids are widely used as preservatives in the production of various food products (Crozier- Dodson et al., 2005). Organic acids as well as their salts also have antilisterial effects, although the efficacy of the organic acids has been reported to be dose dependent and that combinations of

organic acids may be required to increase the antimicrobial action. It has, however, also been found that *L. monocytogenes* is capable of surviving at refrigerated storage even in the presence of additives (Zheng Lu et al., 2005). Storage temperatures lower than 4˚C are, therefore, often necessary for safe storage even when surface treatments utilising organic acid salts are used.

The aim of this study was to determine the susceptibility of *Listeria* spp. isolated from various food premises (Chapter 2) to a range of antimicrobial agents, which include antibiotics used in current treatment regimes for *Listeria* infections as well as organic acids commonly used in food preservation and to determine possible associations between susceptibility to antibiotics and susceptibility to the organic acids.

3.2 MATERIALS AND METHODS

3.2.1 Antibiotics

An agar dilution method as described by the Clinical and Laboratory Standard Institute (CLSI, 2006) was used to determine the minimum inhibitory concentrations (MICs) of all the *Listeria* strains isolated (Chapter 2). Standard antibiotic powders were obtained from Sigma Aldrich (Kemptonpark, RSA) and are listed in Table 3.1. MIC test strips were obtained from Davies Diagnostics (Johannesburg, RSA) and were included if an antibiotic powder was not available. Susceptibility breakpoints as determined by the CLSI were available for only three antibiotics.

Table 3.1 Antibiotics included in MIC determination.

Antibiotic	Abbreviation	Susceptibility breakpoints for *L. monocytogenes* (µg/ml)
Amikacin	AMK	
Ampicillin	AMP	$\leq$ 2
Ciprofloxacin	CIP	
Erythromycin	ERY	
Gentamicin	GEN	
Levofloxacin	LEV	
Penicillin G	PEN	
Tetracycline	TET	$\leq$ 2
MIC test strip		
Clindamycin	CD	0.5 - 4
Imipenem	IMI	
Trimethoprim-sulfamethoxazole	TS	

Logarithmic growth phase cultures were prepared on *Listeria* agar plates incubated for 24 h at 35˚C. A cell suspension was prepared in brain heart infusion (BHI) broth and incubated at 35˚C for 24 h. Cultures were standardised to a McFarland 0.5 (10^7 CFU.ml^{-1}) in saline and diluted 1/10 in 4.5 ml of fresh sterile saline (0.85% NaCl). Cell suspensions were inoculated onto the surface of BHI agar plates containing two-fold antibiotic concentrations, ranging from 0.25–256 µg/ml. A multipoint inoculator (Multipointelite, Mast Diagnostics, UK) was used to deliver a final cell concentration of 1 x 10^5 CFU.ml^{-1} per spot. MICs were read after 24 h incubation at 35˚C and recorded as the lowest concentration of antibiotic that inhibited growth, disregarding one or two colonies or a trailing haze of growth. A control strain, *Streptococcus pneumonia* ATCC

49619 was included in each series. Approved and tentative CLSI susceptible breakpoints (CLSI, 2006) and preliminary breakpoints were used.

MIC tests strips were applied onto a BHI agar surface inoculated with a 0.5 McFarland broth of the test strains and the preformed exponential gradient of antimicrobial agent immediately diffused into the agar matrix. After 18 h incubation at 35˚C a symmetrical inhibition ellipse was formed, centered along the strip. The MIC was read directly from the scale in terms of µg/ml at the point where the edge of the inhibition ellipse intersects the MIC test strip.

3.2.2 Organic acids

The same method as described above was used for the determination of MICs of nine organic acids and five salts obtained from MP Biomedicals, Inc. (Solon, Ohio, USA) and Sigma Chemicals (St Louis, MO, USA). Organic acids included acetic acid, benzoic acid, butyric acid, citric acid, formic acid, lactic acid, malic acid and propionic acid, while the salts consisted of sodium citrate, sorbic acid, potassium sorbate, sodium benzoate and sodium propionate. Concentrations ranged from 0.5 – 300 mM, consisting of 13 dilutions. Dilutions differed because of the molecular weight and solubility of the substance. MICs were performed at pH 5 and pH 7 to determine the variance in susceptibility at different pH levels. The pH of the BHI agar was adjusted before autoclaving. Control strains *L. monocytogenes* (ATCC 19111), *L. welshimeri* (ATCC 35897) and *L. innocua* (ATCC 33090) were included in the MIC determination.

3.3 RESULTS AND DISCUSSION

3.3.1 Antibiotic susceptibility

Overall antibiotic susceptibility patterns of all the isolates indicated high level susceptibility among the majority of the *Listeria* spp. to all the antibiotics tested. Most of the isolates were equally susceptible or more susceptible than the control strain. however, some notable variations did occur and it would be necessary to highlight and discuss these concerns.

For example, one *L. innocua* strain (*L. innocua* 172), showed exceptionally high MIC values for amikacin (>256 µg/ml), gentamicin (64 µg/ml) and trimethoprim-sulfamethoxazole (>32 µg/ml) (Table A.1 – please refer to Appendix Table A.1 for more explanatory MIC results with regard to individual *L. innocua* strains). Elevated MICs were also found for ampicillin (4 µg/ml), ciprofloxacin (2 µg/ml) and clindamycin (0.75 µg/ml), when compared with the control strain (2, 0.5 and 0.19 µg/ml respectively) (Table 3.2). Six of the remaining seven *L. innocua* strains had a higher MIC for gentamicin (2-4 µg/ml) than the control strain (1 µg/ml). All the other *L. innocua* isolates had a higher MIC for ciprofloxacin (1-2 µg/ml) than the control strain (0.5 µg/ml). Three isolates showed very high MICs for trimethoprim-sulfamethoxazole and imipenem (>32 µg/ml, or no zone of inhibition detected). It is interesting to note that the highly resistant *L. innocua* isolate 172 had a much lower MIC for levofloxacin (≤0.25 µg/ml) than any of the other isolates or control strains, irrespective of the species.

Table 3.2: Antibiotic susceptibility ranges for *Listeria innocua* isolates (n = 8) compared to the control strain.

Antibiotic	**MIC (µg/ml)**	
	Isolated strains	***L. innocua*** **ATCC 33090**
Amikacin	4->256	8
Ampicillin	1-4	2
Ciprofloxacin	≤0.25	0.5
Clindamycin	0.5-0.75	0.19
Erythromycin	≤0.25	≤0.25
Gentamicin	1-64	1
Imipenem	0.75->32	>32
Levofloxacin	≤0.25-2	2
Penicillin	≤0.25	≤0.25
Tetracycline	0.5-1	1
Trimethoprim-sulfamethoxazole	0.125-32	>32

The unusually wide range of MIC values observed in Table 3.2 is attributed to only one isolate *L. innocua* 172, which showed extremely high values as opposed to the other seven *L. innocua* isolates.

In Table 3.3 the antibiotic susceptibility of *L. monocytogenes* strains are summarised. For a more detailed representation of the MICs for individual strains, please refer to Appendix Table A.2. One *L. monocytogenes* isolate (*L. monocytogenes* 138) had an MIC of 12 µg/ml for clindamycin, while the MIC for the control strain was 0.5 µg/ml (Table 3.3). Two *L. monocytogenes* isolates (*L. monocytogenes* 77 and 126) also showed increased MIC values for gentamicin (2-4 µg/ml) as opposed to 1 µg/ml of the control strain. All the *L. monocytogenes* isolates were susceptible to imipenem, while the control strain showed high level resistance to imipenem (no zone of inhibition detected).

Table 3.3: Antibiotic susceptibility ranges for *Listeria monocytogenes* isolates (n = 6) compared to the control strain.

	MIC (µg/ml)	
Antibiotic	**Isolated strains**	***L. monocytogenes* ATCC 19111**
Amikacin	4-8	8
Ampicillin	0.5-2	2
Ciprofloxacin	0.5-1	0.5
Clindamycin	0.25-12	0.5
Erythromycin	≤0.25	≤0.25
Gentamicin	1-4	1
Imipenem	0.5-1	>32
Levofloxacin	1-2	2
Penicillin	≤0.25	≤0.25
Tetracycline	≤0.25-1	1
Trimethoprim-sulfamethoxazole	0.032-0.094	>32

All the *L. welshimeri* isolates had a higher MIC for tetracycline (0.5-1 µg/ml) than the control strain (≤0.25 µg/ml), while 13/36 (36%) of the *L. welshimeri* isolates had a higher MIC for gentamicin (2 µg/ml) compared to the control strain (Table 3.4) (For a more detailed representation of the MICs for individual strains, please refer to Appendix Table A.3). One of the *L. welshimeri* isolates (*L. welshimeri* 76) had an MIC of 4 µg/ml for levofloxacin opposed to the MIC of 2 µg/ml of the control strain. All the *L. welshimeri* isolates were susceptible to imipenem, while the control strain showed high level resistance to imipenem (no zone of inhibition detected).

Table 3.4: Antibiotic susceptibility ranges for *Listeria welshimeri* isolates (n = 36) compared to the control strain.

	MIC (µg/ml)	
Antibiotic	**Isolated strains**	***L. welshimeri* ATCC 35897**
Amikacin	4-8	8
Ampicillin	0.5-2	2
Ciprofloxacin	0.5-1	1
Clindamycin	0.25-1	1.5
Erythromycin	≤0.25	≤0.25
Gentamicin	1.2	1
Imipenem	0.25-1.5	>32
Levofloxacin	1.4	2
Penicillin	≤0.25	≤0.25
Tetracycline	0.5-1	≤0.25
Trimethoprim-sulfamethoxazole	0.016-0.094	>32

L. monocytogenes, and also strains of other *Listeria* spp., are generally susceptible to a wide range of antibiotics and the treatment of choice for listeriosis include ampicillin or penicillin G in combination with an aminoglycoside, such as gentamicin, while co-trimoxazole (trimethoprim combined with sulfamethoxazole) is administered as alternative therapy (Charpentier and Courvalin, 1999). In the current study, reduced susceptibility in *L. monocytogenes* was found only against gentamicin (two isolates, one from the rinsing water at the chicken abattoir and one from sushi) and clindamycin (one isolate from sushi). Except for the single *L. innocua* strain with notably high MIC values, and three *L. innocua* isolates resistant to trimethoprim-sulfamethoxazole and imipenem (all from dairy products) antibiotic susceptibility is still intact for all the isolates. The highest overall resistance was noted against amikacin, gentamicin and trimethoprim-sulfamethoxazole. An interesting observation was the high-level resistance of *L. monocytogenes* ATCC 19111 against imipenem, while all the isolated strains were notably susceptible.

Although it is reported that most *Listeria* spp., from clinical as well as food-borne and environmental sources, are susceptible to the antibiotics active against Gram-positive bacteria, the first resistant strains of *L. monocytogenes* were already reported in 1988. These strains were resistant to >10 µg/ml tetracycline (Charpentier and Courvalin, 1999). Tetracycline resistance is in fact, reported to be the most frequently resistance encountered in *L. monocytogenes* isolated from humans. Incidentally tetracycline resistance is also increasing in *Listeria* spp. isolated from food and environmental

sources (Charpentier and Courvalin, 1999). In the current study the MIC levels remained low for all the isolates and control strains.

The treatment of choice for systemic listeric infections in humans include high doses of amoxicillin, with an additional dose of gentamicin for non-pregnant adults (Hof, 2004). However, *L. monocytogenes* is able to grow intracellularly (Havell, 1986) and because aminoclygosides penetrate cells poorly, this agent may be ineffective. For patients with hypersensitivity to penicillin, a combination of trimethoprim-sulfamethoxazole has proved effective in patients with listeriosis (Armstrong, 1995). Antimicrobial agents such as chloramphenicol or tetracycline have also been associated with high treatment failure rates and are therefore not recommended (Southwick and Purich, 1996).

3.3.2 Organic acid susceptibility

Susceptibility to all the organic acids was notably reduced at pH 7 in all the isolates and control strains, especially for the salts potassium sorbate, sodium benzoate and sodium propionate (Figure 3.1). All the isolates and control strains were resistant to sodium citrate at pH 5 as well as pH 7.

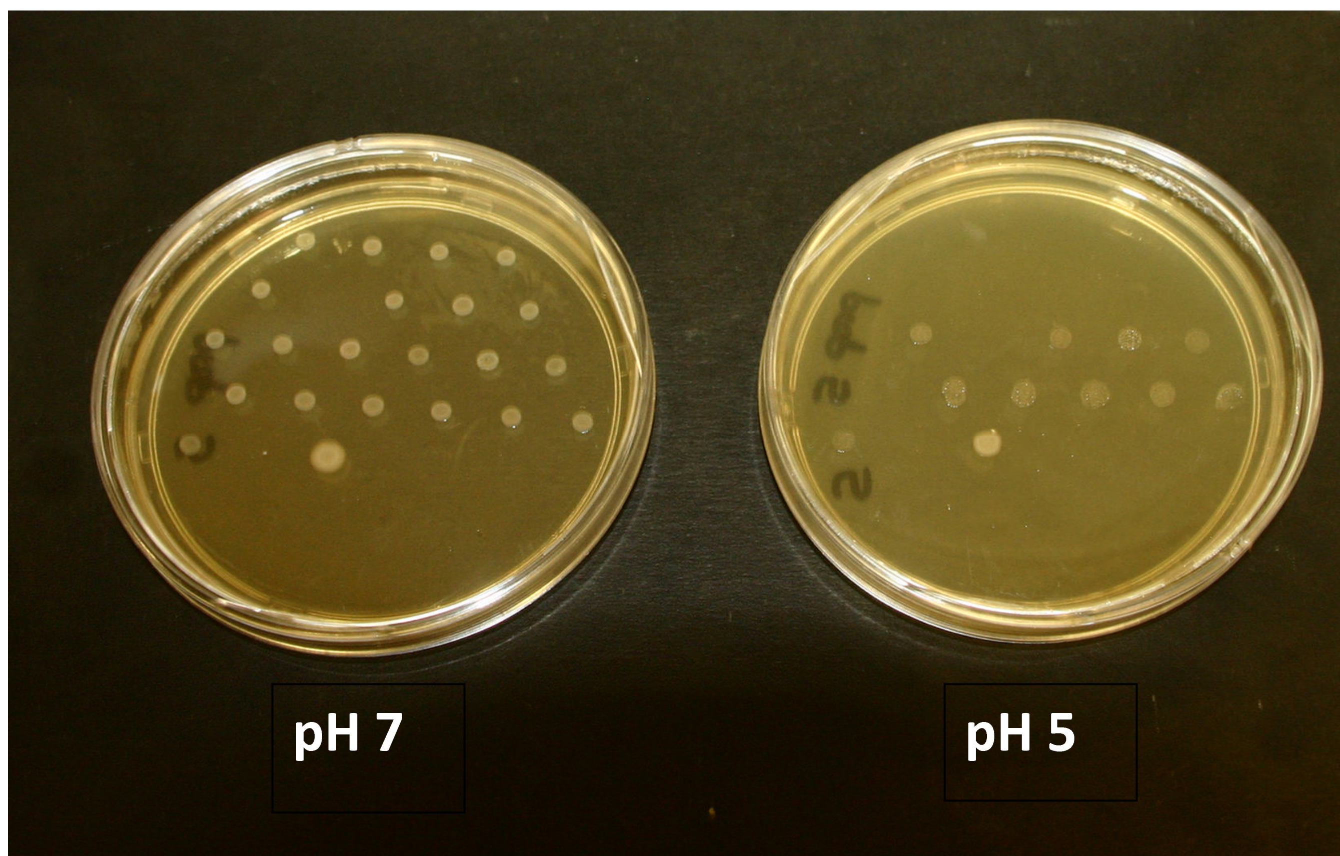

Figure 3.1. Comparison of MICs obtained at two pH values (pH 7 and pH 5) for propionic acid. It is clear that inhibitory activity is higher at pH 5.

In Table 3.5 the organic acid susceptibility at pH 5 and pH 7 of all the *L. monocytogenes* strains are summarised. For a more detailed representation of the organic acid MICs for individual strains at pH 5 and pH 7 respectively, please refer to Appendix Table A.4 and A.5. Higher MICs were found at pH 5 for lactic acid in all isolates (10 mM) compared to the control strain (5 mM). One isolate (*L. monocytogenes* 77) had a higher MIC for benzoic acid at pH 5 (10 mM) (Table A.4) than the control strain (5 mM). Two isolates (*L. monocytogenes* 60 & 77) had higher MICs at pH 5 for sodium benzoate (20

mM) and sodium propionate (20 mM) (Table A.4) than the control strain (10 mM) (Table 3.5). One isolate (*L. monocytogenes* 126) had a distinctly high MIC of 150 mM for sodium propionate at pH 5 (Table A.4). All the *L. monocytogenes* isolates were equally or more susceptible at pH 5 than the control strain for acetic acid, butyric acid, citric acid, fumaric acid, malic acid, potassium sorbate, propionic acid and sorbic acid.

Organic acid susceptibility of all the *L. welshimeri* isolates compared to the control strain at pH 5 and pH 7 is presented in Table 3.6. For a more detailed representation of the organic acid MICs for individual strains at pH 5 and pH 7 respectively, please refer to Appendix Table A.6 and A.7. All the *L. innocua* isolates were equally or more susceptible at pH 5 than the control strain for butyric acid, citric acid, fumaric acid, lactic acid, malic acid, propionic acid, and sorbic acid. At pH 5, 27 (75%) of the *L. welshimeri* isolates were less susceptible than the control strain to acetic acid (MIC 2.5-5 mM), 12 (33.33%) were less susceptible to benzoic acid (MIC 10 mM), while 24 (66.7%) isolates had four times the MIC (20 mM) of that of the control strain (MIC 5 mM). Only one of the *L. welshimeri* isolates (*L. welshimeri* 85) was less susceptible for sodium propionate (MIC 50 mM) than the control strain (MIC 20 mM) (Table A.6).

Table 3.5: Susceptibility of *Listeria monocytogenes* isolates to organic acids (n = 6) compared to the control strain.

Organic acid	**MIC (mM)**			
	Isolated strains		***L. monocytogenes* ATCC 19111**	
	pH 5	**pH 7**	**pH 5**	**pH 7**
Acetic acid	2.5-5	25-50	5	25
Benzoic acid	1-10	20-30	5	30
Butyric acid	5-10	25-50	10	50
Citric acid	2.5	20	2.5	20
Formic acid	2.5	20-25	2.5	25
Lactic acid	10	50-100	5	50
Malic acid	2.5-10	20	10	20
Potassium sorbate	10-50	200-300	20	100
Propionic acid	2.5-5	25-50	2.5	20
Sodium benzoate	5-20	150	5	150
Sodium citrate	100->150	>150	150	>150
Sodium propionate	5-150	250-300	10	300
Sorbic acid	5-10	20->30	10	20

Table 3.6: Susceptibility of *Listeria welshimeri* isolates to organic acids (n = 36) compared to the control strain.

Organic acid	**MIC (mM)**			
	Isolated strains		***L. welshimeri* ATCC 35897**	
	pH 5	**pH7**	**pH 5**	**pH 7**
Acetic acid	≤0.5-5	20-50	1	25
Benzoic acid	1-10	20-30	5	30
Butyric acid	5-10	20-50	10	50
Citric acid	2.5	20	2.5	20
Formic acid	2.5-5	20-25	2.5	25
Lactic acid	5-10	50	10	50
Malic acid	2.5-10	20	10	20
Potassium sorbate	2.5-20	200-300	20	200
Propionic acid	2.5	25	2.5	25
Sodium benzoate	≤0.5-20	100-150	5	150
Sodium citrate	>150	150->150	>150	>150
Sodium propionate	5-50	150-300	20	300
Sorbic acid	5-10	20->30	10	20

In Table 3.7 organic acid susceptibility of all the *L. innocua* isolates are compared to the control strain at pH 5 and pH 7. For a more detailed representation of the organic acid MICs for individual strains at pH 5 and pH 7 respectively, please refer to Appendix Table A.8 and A.9. At pH 5 all the *L. innocua* isolates were equally or more susceptible than the control strain for benzoic acid, butyric acid, malic acid, potassium sorbate, sodium benzoate, sodium propionate and sorbic acid (Table A.8). All the *L. innocua* isolates had an MIC for lactic acid (10 mM), which is twice that of the control strain (5

mM), while seven (87.5%) isolates were less susceptible to propionic acid (MIC 5-10 mM) than the control strain (MIC 2.5 mM). Three (37.5%) isolates had an MIC for acetic acid five times (25 mM) than that of the control strain (5 mM). One isolate (*L. innocua* 153) had an MIC of 50 mM as opposed to that of the control strain (20 mM).

Table 3.7: Susceptibility of *Listeria innocua* isolates to organic acids (n = 8) compared to the control strain.

Organic acid	**MIC (mM)**			
	Isolated strains		***L. innocua* ATCC 33090**	
	pH 5	**pH7**	**pH 5**	**pH 7**
Acetic acid	1-25	25-50	5	50
Benzoic acid	1-10	20-30	10	30
Butyric acid	5-10	25-50	10	50
Citric acid	2.5-5	20-25	2.5	20
Formic acid	2.5-5	20-25	5	25
Lactic acid	10	50	5	50
Malic acid	5-10	20	10	20
Potassium sorbate	10-50	150-300	20	200
Propionic acid	2.5-10	25-50	2.5	25
Sodium benzoate	5-20	150	20	150
Sodium citrate	>150	>150	>150	>150
Sodium propionate	5-20	150-300	20	300
Sorbic acid	5-10	20->30	10	20

Reduction of organic acid activity at higher pH values was evident against all the *Listeria* isolates in the study. Organic acids are weak acids with pK_a (ionisation constant) ranges between pH 3 and 5 (Doores, 1993; Fang and Tsai, 2003) and are most active at a pH equal or lower than their *p*K_a value (Brul et al*.,* 2002). At low pH values weak acids are mainly in their un-dissociated form and it is in this state that they can then freely diffuse across the bacterial cell membrane and enter the cytosol (Papadimitriou et al., 2007). As the pH value increases the amount of un-dissociated acid also decreases (Price-Carter et al., 2005).

Decreased susceptibility was evident in some *L. monocytogenes* strains for benzoic acid, sodium benzoate and sodium propionate, while all six strains indicated a slight decrease in susceptibility for lactic acid. *In vitro* studies have shown that the inhibitory action of organic acids on *L. monocytogenes* depends on the characteristics of the acid, such as the pK_a value (dissociation constant) (Buchanan and Klawitter, 1990; Glass et al., 1992; Rayboudi-Massilia et al., 2009). For example, acetic acid and propionic acid both have higher pK_a values than lactic acid and, as a result they possess a higher proportion of un-dissociated acid at a specific pH (Schnürer and Magnusson, 2005). It would, therefore, be expected that they have a greater inhibitory effect than lactic acid. *L. monocytogenes* have also been reported to use lactate as a source of carbohydrate (Kouassi and Shelef, 1996).

Previous studies have found *L. monocytogenes* to be more sensitive to organic acids than Gram-negative bacteria such as *Salmonella enteritidis* or *Escherichia coli*

O157:H7. This may be attributed to the Gram-positive cell wall, which contains only a thick peptidoglycan layer and a lipid bilayer (Rayboudi-Massilia et al., 2009). This cell membrane is therefore, generally impermeable to polar compounds. Malic acid, for example, has low lipid solubility, and this would limit entry into the cell. Effectiveness of organic acids also varies depending on its molecular weight. The smaller molecules of malic acid (134.09 Da) and lactic acid (90.08 Da) may enter into the bacterial cells more easily than the larger molecules of citric acid (192.13 Da) and tartaric acid (150.09 Da) (Rayboudi-Massilia et al., 2009).

Although the optimum pH for *L. monocytogenes* is 7-8, this organism may grow in a pH range of 5-10 (Sorrels et al., 1989). A number of studies have also shown that *L. monocytogenes* is more acid tolerant than most food-borne pathogens. However, the sensitivity of the organism to organic acids varies with the nature of the acidulant used as well as the concentration of organic acid in food products. The concentration of organic acids used in the industry is also dependent on the type of food. Although organic acids have been reported to produce adverse sensory changes, dilute solutions of organic acids (1-3%) are generally applied without any effect on sensory properties of meat (Smulders and Greer, 1998; Min *et al*., 2007). Good Manufacturing Practices (GMP) standard is recognised as an absolutely essential standard to reduce risk of food safety and quality deviations. GMP is also a pre-requisite to implementing HACCP standards in companies. In South Africa the SABS 049 code of practice was implemented and is followed to achieve good practices.

In one *L. innocua* strain (isolate 172) an indirect relationship was found between the susceptibility to the organic acids and susceptibility to antibiotics (Appendix Table A.3 and A.8) This strain was highly resistant to amikacin, ampicillin, gentamicin, imipenem and trimethoprim/sulphamethoxazole (Table A.3), yet highly sensitive to all the organic acids (Table A.8). It would be interesting and revelatory to investigate possible mechanisms involved in resistance development against antibiotics as well as the organic acids.

3.4 CONCLUSIONS

Although antimicrobials used as growth promoters in animal feed have caused a decrease in infectious diseases, such as diarrhea, abscesses and mastitis, it has resulted in the spreading of antimicrobial-resistant *L. monocytogenes* into the environment. It has, therefore, become imperative that antimicrobial resistance of *L. monocytogenes* in humans and animals be monitored to recognise alterations in susceptibility to antimicrobials commonly used, to apply control measures for the use of these antimicrobial agents and to prevent the spread of multi-resistant strains (Harakeh et al., 2009).

Susceptibility of the isolates from samples was often lower than the reference strains, but MIC values of the organic acids also indicated much higher susceptibility at the lower pH value (pH 5). Organic acids are known to have better activity at a lower pH and should, therefore, be more inhibitory in acidic foodstuff when applied as food

preservatives. However, *Listeria* is an organism notorious of growing at extremely low pH values and continuous monitoring of foodstuff should be imperative.

3.5 REFERENCES

Armstrong, D., 1995. *Listeria monocytogenes.*in G.L. Mandell, J.E. Bennett and R. Dolin (Ed.). Principles and Practice of Infectious diseases (4th ed.). Churchill Livingstone, NY, pp 1880-1885.

Brul, S., Klis, F.M., Oomes, S.J.C.M., et al. 2002. Detailed process design based on genomics of survivors of food preservation processes. *Trends in Food Science and Technology,* 13:325–333.

Buchanan, R.L. and Klawitter, L.A. 1990. Effects of temperature and oxygen on the growth of *Listeria monocytogenes* at pH 4.5. *Journal of Food Science,* 55:1754-1756.

Charpentier, E. and Courvalin, P. 1999. Antibiotic resistance in *Listeria* spp. *Antimicrobial Agents and Chemotherapy,* 43:2103-2108.

Clinical and Laboratory Standards Institute, 2006a. Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria. Vol. 26, no. 19, M45-A. Approved guideline. Wayne, P.A.: Clinical and Laboratory Standards Institute.

Crozier-Dodson, E.A., Carter, M. and Zheng, Z. 2005. Process control: formulating food safety: an overview of antimicrobial ingredients. *Food Safety Magazine***,** Dec 2004/Jan 2005.

Doores, S. 1993. Organic acids. In: A.L. Branen and P.M. Davidson (Eds.), *Antimicrobials in Foods* 2nd ed., pp.95–136. New York: Marcel Dekker.

Fang, T.J. and Tsai, H.C. 2003. Growth patterns of *Escherichia coli* O157:H7 in ground beef treated with nisin, chelators, organic acids and their combinations immobilized in calcium alginate gels. *Food Microbiology,* 20:243–253.

Faleiro, M.L., Andrew, P.W. and Power, D. 2003. Stress response of *Listeria monocytogenes* isolated from cheese and other foods. *International Journal of Food Microbiology,* 84:207-216.

Ghandi, M. and Chikindas, M.L. 2007. *Listeria*: A food borne pathogen that knows how to survive. *International Journal of Food Microbiology***,** 113:1–15.

Glass, K., Loeffelhoz, J., Ford, J. and Doyle, M. 1992. Fate of *Escherichia coli* 0157:H7 as affected by pH or sodium chloride in fermented, dry sausage. *Applied and Environmental Microbiology,* 58:2513-2516.

Harakeh, S., Saleh, I., Zouhairi, O., Baydoun, E., Barbour, E. and Alwan, N. 2009. Antimicrobial resistance of *Listeria monocytogenes* isolated from dairy-based food products. *Science of the Total Environment,* 407:4022-4027.

Havell, E.A. 1986. Synthesis and secretion of interferon by urine fibroblasts in response to intra-cellular *Listeria monocytogenes. Infection Immunology,* 54:787-792.

Hof, H. 2004. An update on the medical management of listeriosis. *Expert Opinion Pharmacotherapy*, 5:1727-1735.

Kouassi, Y. and Shelef, L.A. 1996. Metabolic activities of *Listeria monocytogenes* in the presence of sodium propionate, acetate, lactate and citrate. *Journal of Applied Bacteriology,* 81:147-153.

Min, J.S., Lee, S.O., Jang, A., Jo, C. and Lee, M. 2007. Irradiation and organic acid treatment for microbial control and the production of biogenic amines in beef and pork. *Food Chemistry,* 104:791-799.

Papadimitriou, M.N., Resende, C., Kuchler, K. and Brul, S. 2007. High Pdr12 levels in spoilage yeast (*Saccharomyces cerevisiae*) correlate directly with sorbic acid levels in the culture medium but are not sufficient to provide cells with acquired resistance to the food preservative. *International Journal of Food Microbiology,* 113:173-179.

Price-Carter, M., Fazzio, T.G., Vallbona, E.I. and Roth, J.R. 2005. Polyphosphate kinase protects *Salmonella enterica* from weak organic acid stress. *Journal of Bacteriology,* 187:3088-3099.

Raybaudi-Massilia, R.M., Mosqueda-Melger, J. and Martin-Belleso, O. 2009. Antimicrobial activity of malic acid against *Listeria monocytogenes, Salmonella enteritidis* and *Escherichia coli* 0157:H7 in apple, pear and melon juices. *Food Control***,** 20:105-112.

Schnürer, J. and Magnusson, J. 2005. Antifungal lactic acid bacteria as bio-preservatives. *Trends in Food Science and Technology,* 16:70-78.

Smulders, F.J. and Greer, G.G. 1998. Integrating microbial decontamination with organic acids in HACCP programmes for muscle foods: prospects and controversies. *International Journal of Food Microbiology*, 44:149-169.

Sorrels, K.M., Enigl, D.C. and Harfield, J.R. 1989. Effect of pH, acidulant, time and temperature on the growth and survival of *Listeria monocytogenes. Journal of Food Protection,* 52:571-573.

Southwick, F.S. and Purich, D.L. 1996. Intracellular pathogenesis of listeriosis. *New England Journal of Medicine***,** 334:770-776.

Zheng Lu, Sebranek, J.G., Dickson, J.S., Mendonca, A.F. and Bailey, T.B. 2005. Inhibitory effects of organic acid salts for control of *Listeria monocytogenes* on frankfurters. *Journal of Food Protection*, 68:499-506.

CHAPTER 4

EXPOSURE OF *LISTERIA* SPP. TO SUB-LETHAL TREATMENT OF ANTIMICROBIAL AGENTS

4.1 INTRODUCTION

In animal feed, antimicrobials used as growth promoters have reduced the impact of infectious diseases such as diarrhea, but has also led to the dissemination of antimicrobial-resistant *Listeria monocytogenes* into the environment (Teuber, 1999). Antimicrobial-resistant bacteria in the environment have emerged due to the excessive use of antimicrobials. It is therefore important to monitor the antimicrobial resistance patterns of *L. monocytogenes* in humans as well as in animals. The first reported resistant *L. monocytogenes* strains were resistant to >10 µg/ml of tetracycline, and this was already in 1988 (Poyart-Salmeron et al., 1990).

The presence of a resistance plasmid in *L. monocytogenes* may be implicated in antimicrobial resistance as genes may be transferred by conjugation. Mutational events in chromosomal genes can also play a role in exchange of resistance between *Listeria* species (Poros-Gluchowska and Markiewicz, 2003). Antibiotic resistance is increasingly reported widely in all bacteria, not only in pathogens, and poses a risk to human health. Many antibiotic-resistant bacteria in foods are saprophytic, but their resistance genes can be transferred to other food-borne bacteria, including pathogenic species within the gastrointestinal tract (Perreten et al., 1997). This may have undesirable clinical implications for the host, and for the rest of the population that comes into contact with the antibiotic-resistant pathogens.

L. monocytogenes has the ability to respond and adapt to changing environments, thus making it difficult to control the organism in food environments. US regulatory agencies, such as the FDA have established a zero tolerance policy for *L. monocytogenes* in RTE foods because of an increase in the number of listeriosis outbreaks associated with these food products (Jay, 2000). Consumers have ever-changing food habits and there is a trend towards consumption of minimally processed, ready-to-eat convenience foods and refrigerated or frozen food products. These habits have affected the incidence of listeriosis (Rocourt and Bille, 1997). There are indications that microorganisms will increasingly become resistant to preservatives such as the organic acids, the same take place in human pathogens as they become increasingly resistant to antibiotics (Theron and Lues, 2007). Mechanisms of survival under adverse environmental conditions include survival at low temperatures, survival under acid stress and survival under osmotic stress

The aim of this study was, therefore, (1) to monitor the induction of resistance development against food preservatives such as organic acids as well as antibiotics used in the current treatment regime for *Listeria* infections, (2) to determine the possibility of cross-resistance against antibiotics and organic acids and (3) to determine the involvement of cell proteins in resistance development.

4.2 MATERIALS AND METHODS

4.2.1 Induction

Eight highly susceptible strains were selected for induction from each of the isolated species. These isolates as well as the three control strains *L. monocytogenes* ATCC 19111, *L. welshimeri* ATCC 35897 and *L. innocua* ATCC 33090 were exposed to increasing concentrations of three antibiotics (penicillin, tetracycline, ciprofloxacin) and three organic acids (citric acid, acetic acid and lactic acid). The first concentration used was one concentration below the MIC: Citric acid 10 mM, acetic acid 20 mM, lactic acid 20 mM, penicillin 0.125 µg/ml, tetracycline 0.25 µg/ml and ciprofloxacin 0.25 µ/ml. Induction was performed in 5 ml brain heart infusion (BHI) broth. One hundred microliters of an overnight broth culture of the specific strains were inoculated into the broth containing the inducing agent. The tubes were incubated on a platform shaker at 35˚C at 120 rpm and observed daily for growth. These cultures were streaked out on BHI agar containing relevant concentrations of the inducing agent in order to maintain developed resistance. Growth from these plates was used for protein analysis. Induction was terminated at the highest concentration where growth could be detected.

4.2.2 Susceptibility Testing

Minimum inhibitory concentrations of all the induced strains were determined for the penicillin, ampicillin, tetracycline, gentamicin, ciprofloxacin, acetic acid, citric acid and lactic acid as described in Chapter 3. MIC determination of the organic acids was performed at pH 5 only.

4.2.3 Protein Extraction

Preparation of protein samples

After induction, overnight cultures were harvested by centrifugation at 12 000 x g for 5 min (Eppendorf-Netheler-Hinz). Cells were washed twice with sodium phosphate buffer (0.01 M, pH 7). Washed cells were suspended in 900 µl sample treatment buffer (distilled H_2O, 0.5 M Tris-HCl, pH 6.8 [Saarchem, Merck Chemicals [PTY] Ltd., Modderfontein, RSA], glycerol [Roche Diagnostics Corporation, Isando, RSA], 10% [w/v] SDS, 2 β-mercaptoethanol [MP Biochemicals Inc., Menlopark, RSA], 0.05% [w/v] bromophenol blue [Saarchem]) in eppendorf tubes. The suspension was mixed thoroughly by vortexing and 100 µl EDTA (0.05 M) and 100 µl lysozyme (Roche Diagnostics Corporation) solution added. Tubes were incubated for 30 min at 37˚C. After incubation, 100 µl of 20% SDS (Saarchem) was added and the solution was mixed again. The suspension was heated at 95˚C for 10 min, allowed to cool and centrifuged at 10 000 x g for 10 min. The supernatant was stored in eppendorf tubes at -20˚C until protein separation by SDS-PAGE.

SDS PAGE

Protein samples were loaded onto a 12% discontinuous SDS-PAGE slab gel, consisting of a stacking gel (0.5 M Tris-HCl [pH 6.8], 10% [wt/wt] SDS, 30% [wt/vol] acrylamide/bisacrylamide [Saarchem], 10 % [wt/vol] ammonium persulphate [Saarchem], TEMED [Saarchem]), and a separating gel (1.5 M Tris-HCl [pH 8.8], 10% [wt/wt] SDS, 30% [wt/vol] acrylamide/bisacrylamide, 10% [wt/vol] ammonium persulphate, TEMED). Gels were run in a PROTEAN II xi cell (Bio-Rad, Johannesburg, RSA) attached to a Haake K10 cooling system (Lasec, Bloemfontein, RSA) at a constant current of 16 mA per gel through the stacking gel and 24 mA per gel through the separating gel. Gels were run in a tris-glycine running buffer (25 mM Tris, 192 mM glycine [Saarchem], 0.1% SDS), pH 8.3, with a running time between 4 to 5 hours. Gels were stained with 0.1% Coommassie brilliant blue (Saarchem) in 40% ethanol (Saarchem) and 10% acetic acid (Saarchem) and destained with 40% methanol (Saarchem) and 10% acetic acid. Protein profiles were captured with a GelDoc XR (Bio-Rad) and molecular weight determined by Quantity One® 1-D Analysis Software (Bio-Rad).

4.3 RESULTS AND DISCUSSION

Minimum inhibitory concentrations after induction showed notable variations and are presented in Tables 4.1 to 4.3. Depicted on the tables are MICs of ampicillin, ciprofloxacin, gentamicin, penicillin, tetracycline, acetic acid, citric acid and lactic acid for induced strains. Only the strains that were able to survive at least one induction step are included in the tables. The boxed values show an increase in MIC of two or more doubling concentrations in the induced strain.

Induction with antibiotics as well as organic acids did not result in general resistance development. However, a few isolated cases of reduced susceptibility were found in all of the induced strains, except *L. monocytogenes* ATCC 19111, although no definite pattern could be observed (Table 4.1). Overall increased MIC values were demonstrated in tetracycline and ciprofloxacin after induction with the respective agents.

Induction with acetic acid resulted in a notable increase in MIC values of ampicillin for *L. monocytogenes* 138 and 139 (1 to 4 µg/ml), while induction with citric acid similarly caused an MIC increase of ampicillin in *L. monocytogenes* 139 (1 to 4 µg/ml). Induction with acetic acid as well as lactic acid also resulted in a notable increase in MIC values of ciprofloxacin for *L. monocytogenes* ATCC 19111 (0.5 to 2 µg/ml). In addition, induction with tetracycline also caused an increase in MIC value of acetic acid for *L. monocytogenes* 139 (2.5 to 10 µg/ml). Such cross induction of resistance may play a

Table 4.1 Comparison of inhibitory concentrations of five antibiotics and three organic acids after induction of three *Listeria monocytogenes* and *L. monocytogenes* ATCC19111.

	MIC (µg/ml) before and after induction										MIC (mM) before and after induction					
	Ampicillin		Ciprofloxacin		Gentamicin		Penicillin		Tetracycline		Acetic acid		Citric acid		Lactic acid	
	before	after	before	After	before	after	before	after	before	after	before	after	before	after	before	after
60 CA[ab]	2	2	0.5	0.5	1	1	≤0.25	≤0.25	0.5	1	2.5	5	2.5	2.5	10	10
60 LA	2	4	0.5	1	1	2	≤0.25	≤0.25	0.5	1	2.5	5	2.5	2.5	10	10
60 TE[#]	2	2	0.5	1	1	2	≤0.25	≤0.25	0.5	16	2.5	5	2.5	2.5	10	10
60 CP	2	4	0.5	128	1	2	≤0.25	≤0.25	0.5	2	2.5	5	2.5	2.5	10	10
138 AA	1	4	1	1	1	2	≤0.25	≤0.25	1	1	5	10	2.5	5	10	10
138 LA	1	2	1	1	1	1	≤0.25	≤0.25	1	1	5	10	2.5	5	10	10
138 TE[#]	1	4	1	1	1	2	≤0.25	≤0.25	1	16	5	10	2.5	5	10	10
139 AA	1	4	1	0.5	1	1	≤0.25	≤0.25	1	1	2.5	10	2.5	5	10	10
139 CA	1	4	1	1	1	1	≤0.25	≤0.25	1	1	2.5	10	2.5	5	10	10
139 TE[#]	1	4	1	0.5	1	1	≤0.25	≤0.25	1	16	2.5	10	2.5	5	10	10
ATCC AA	2	2	0.5	2	1	1	≤0.25	≤0.25	1	1	5	10	2.5	5	5	10
ATCC LA	2	4	0.5	2	1	2	≤0.25	≤0.25	1	1	5	10	2.5	2.5	5	10
ATCC TE*	2	2	0.5	1	1	1	≤0.25	≤0.25	1	2	5	10	2.5	2.5	5	10

a Inducing agent: AA = Acetic acid, CA = citric acid, LA = lactic acid, CP = ciprofloxacin, TE = tetracycline.
b Final induction concentrations: acetic acid 40 mM, citric acid 20 mM, lactic acid 40 mM, ciprofloxacin 32 µg/ml, tetracycline 32[#] and 2* µg/ml.

Table 4.2 Comparison of inhibitory concentrations of five antibiotics and three organic acids after induction of four *Listeria welshimeri* and *L. welshimeri* ATCC 35897.

	MIC (µg/ml) before and after induction										MIC (mM) before and after induction					
	Ampicillin		Ciprofloxacin		Gentamicin		Penicillin		Tetracycline		Acetic acid		Citric acid		Lactic acid	
	before	after	before	After	before	after	before	after	before	after	before	after	before	after	before	after
87 LA[ab]	2	2	0.5	1	1	2	≤0.25	≤0.25	0.5	1	≤0.5	5	2.5	5	10	10
87 PE	2	64	0.5	1	1	4	≤0.25	32	0.5	0.5	≤0.5	5	2.5	2.5	10	10
87 TE#	2	4	0.5	0.5	1	2	≤0.25	≤0.25	0.5	16	2.5	5	2.5	2.5	10	10
87 CP	2	4	0.5	128	1	2	≤0.25	≤0.25	0.5	2	≤0.5	5	2.5	2.5	10	10
88 CA	2	4	0.5	1	1	1	≤0.25	≤0.25	0.5	1	≤0.5	5	2.5	5	10	10
88 LA	2	4	0.5	1	1	2	≤0.25	≤0.25	0.5	1	≤0.5	5	2.5	2.5	10	10
88 TE#	2	2	0.5	1	1	2	≤0.25	≤0.25	0.5	16	≤0.5	5	2.5	2.5	10	10
88 CP	2	4	0.5	128	1	2	≤0.25	≤0.25	0.5	2	2.5	5	2.5	2.5	10	10
92 CA	2	4	0.5	2	2	2	≤0.25	≤0.25	0.5	0.5	≤0.5	10	2.5	5	10	10
92 TE#	2	4	0.5	1	2	1	≤0.25	≤0.25	0.5	16	≤0.5	5	2.5	2.5	10	10
92 CP	2	4	0.5	128	2	2	≤0.25	≤0.25	0.5	2	≤0.5	5	2.5	2.5	10	10
95 LA	0.5	4	0.5	0.5	1	1	≤0.25	≤0.25	0.5	1	≤0.5	5	2.5	5	10	10
95 TE#	0.5	4	0.5	1	1	2	≤0.25	≤0.25	0.5	16	≤0.5	5	2.5	2.5	10	10
95 CP	0.5	4	0.5	128	1	2	≤0.25	≤0.25	0.5	2	≤0.5	5	2.5	2.5	10	10
ATCC CA	2	4	1	1	1	1	≤0.25	≤0.25	≤0.25	1	1	10	2.5	5	10	10
ATCC LA	2	4	0.5	1	1	2	≤0.25	≤0.25	0.5	1	2.5	5	2.5	2.5	10	10
ATCC TE*	2	4	1	1	1	1	≤0.25	≤0.25	≤0.25	8	1	10	2.5	2.5	10	10

a Inducing agent: AA = Acetic acid, CA = citric acid, LA = lactic acid, CP = ciprofloxacin, PE = 1µg/ml, TE = tetracycline.
b Final induction concentrations: acetic acid 40 mM, citric acid 20 mM, lactic acid 40 mM, ciprofloxacin 32 µg/ml, tetracycline 32# and 2* µg/ml.

Table 4.3 Comparison of inhibitory concentrations of five antibiotics and three organic acids after induction of one *Listeria innocua* and *L. innocua* ATCC 33090.

	MIC (µg/ml) before and after induction										MIC (mM) before and after induction					
	Ampicillin		Ciprofloxacin		Gentamicin		Penicillin		Tetracycline		Acetic acid		Citric acid		Lactic acid	
	before	after	before	After	before	after	before	after	before	after	before	after	before	after	before	after
105 AA	1	4	1	2	2	2	≤0.25	≤0.25	1	1	5	10	2.5	5	10	20
105 CA[#]	1	4	1	2	2	2	≤0.25	≤0.25	1	0.5	5	10	2.5	5	10	10
105 TE	1	4	1	2	2	2	≤0.25	≤0.25	1	16	5	10	2.5	5	10	10
ATCC AA	2	4	0.5	2	1	2	≤0.25	≤0.25	1	0.5	5	10	2.5	5	5	10
ATCC CA	2	4	0.5	2	1	2	≤0.25	≤0.25	1	0.5	5	10	2.5	5	5	10
ATCC LA	2	4	0.5	2	1	4	≤0.25	≤0.25	1	0.5	5	10	2.5	5	5	10
ATCC TE*	2	4	0.5	2	1	1	≤0.25	≤0.25	1	16	5	10	2.5	5	5	10

a Inducing agent: AA = Acetic acid, CA = citric acid, LA = lactic acid, CP = ciprofloxacin, PE = 1µg/ml, TE = tetracycline.
b Final induction concentrations: acetic acid 40 mM, citric acid 20 mM, lactic acid 40 mM, ciprofloxacin 32 µg/ml, tetracycline 32[#] and 16* µg/ml.

significant role in both the clinical treatment of *Listeria* infections, as well as successful control of *L. monocytogenes* in food products.

Of all induced isolates, *L. welshimeri* 87 was the only strain where induced resistance was demonstrated after induction with penicillin (Table 4.2). This was detected in increased MIC values for ampicillin, gentamicin, penicillin as well as acetic acid, again indicating cross resistance induction between an antibiotic and an organic acid. Such cross resistance induction was also found after induction with tetracycline in *L. welshimeri* isolates 88, 95 and ATCC 35897 and after induction with ciprofloxacin in *L. welshimeri* isolates 87, 92 and 95 (Table 4.2). Although *L. welshimeri* is not a recognised pathogen, development of resistance should be monitored as resistance genes can be transferred to other important food-borne pathogens, such as *L. monocytogenes* (Poyart-Salmeron et al., 1990). In *L. innocua* 105 cross resistance induction was found after induction with acetic as well as citric acid as this led to a notable increase in MIC value for ampicillin (1 to 4 µg/ml) (Table 4.3).

No definite correlation could be observed between protein profiles and induced resistance. In general, in all the induced strains more protein bands were observed after induction with the antibiotics, in particular induction with tetracycline. Only a few well defined protein bands could be detected in all the isolates. It would be necessary to conduct a comparison of total protein profiles to demonstrate the differences between species and serovars in order to identify specific alterations in protein composition as a

result of decreased susceptibility. In Figure 4.1 a photograph of a protein gel is shown to illustrate protein profiles of induced strains.

All protein bands that could be visually detected are presented in detail in Appendix B. Fewer protein bands were visible in all the tetracycline induced strains of *L. welshimeri* (Appendix Tables B.5 to B.9) as well as *L. innocua* (Tables B.10 and B.11) than in the *L. monocytogenes* isolates (Tables B.10 to B.13). Lactic acid induction did not produce less visible protein bands than were observed in *L. monocytogenes* induction protein profiles. In *L. monocytogenes* 60 no definite difference was demonstrated between protein profiles of strains after induction with organic acids (citric and lactic acid) as opposed to antibiotics (ciprofloxacin and tetracycline) (Table B.10). This difference is obvious when observing the band sizes, as more prominent protein bands of organic acid induced strains could be observed in the lower ranges of protein bands, while the strains induced with ciprofloxacin and tetracycline produced more prominent proteins bands of higher molecular weight. Bands that were evidently darker stained, were found in the strains induced by ciprofloxacin and tetracycline, as none of these band were observed in the un-induced strain. No resistance was induced in *L. monocytogenes* ATCC 19111 against any of the antibiotics. Similar protein bands were observed after induction with acetic and lactic acids.

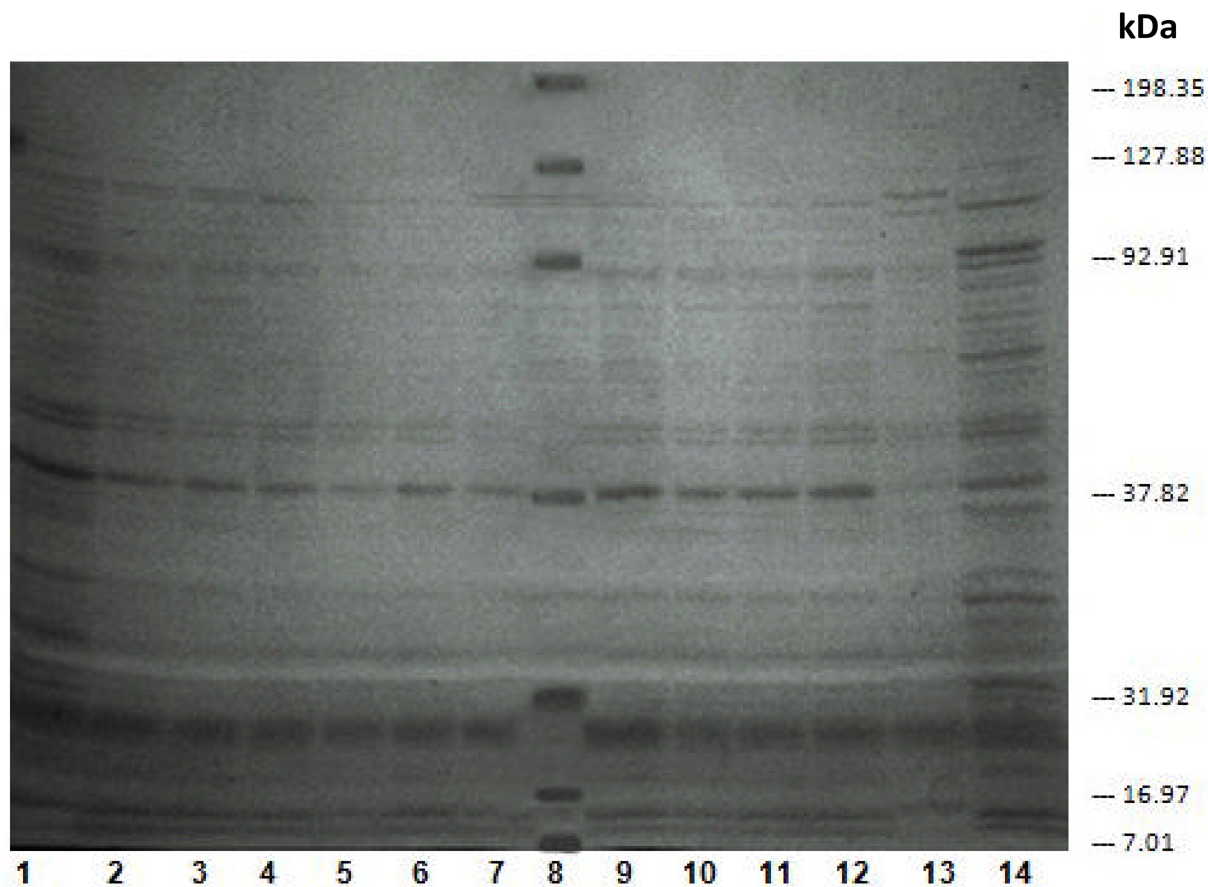

Figure 4.1: Total protein profiles of seven *Listeria* spp. after induction with various antibiotics. Lanes 1-3, *L. welshimeri* 87 (penicillin, ciprofloxacin and tetracycline); lanes 4-5 *L. monocytogenes* 60 (ciprofloxacin and tetracycline); lane 6-7 *L. welshimeri* 88 (ciprofloxacin and tetracycline); lane 8, Molecular Weight Marker (Bio-Rad); lanes 9-10, *L. welshimeri* 92 (ciprofloxacin and tetracycline); lanes 11-12, *L. welshimeri* 95 (ciprofloxacin and tetracycline); lane 13, *L. innocua* 105 (tetracycline); lane 14, *L. monocytogenes* 138 (tetracycline).

In the *L. welshimeri* 87 penicillin induced strain numerous protein bands were visible, as opposed to only a few visible bands in the un-induced strain (Table B.5). In the un-induced sample of *L. welshimeri* 92 (Table B.7), notably more protein bands were visible over a wide size range (5.4 – 85.1 kDa). This was also found in the citric acid induced strain (5.4 – 93.4 kDa).

Numerous protein bands were visible in the un-induced *L. innocua* 105 strain in the lower protein size range (9.8 – 37.3 kDa) (Table B.10). Of interest were the protein bands observed in the tetracycline induced strain in the higher size range (39.6 – 104.3 kDa). In the un-induced strain of *L. innocua* ATCC 33090 only a few protein bands were visible and ranged from 7.4 to 82.3 kDa (Table B.11). Protein bands found in all the induced strains were also visibly distributed over a wide range of protein sizes. Protein could be detected in the samples induced by all three organic acids (acetic, citric and lactic acid). This was not found in any of the induced strains of either *L. monocytogenes* or *L. welshimeri*.

4.4 CONCLUSIONS

The study provided information with regard to the potential of *Listeria* spp. in developing resistance after continuous exposure to antimicrobial agents. This was indicated by a substantial increase in MIC values in various isolates after exposure to different antimicrobial agents. Although the study did not provide sound proof of concurrent resistance development against antibiotics as well as preservatives such as the organic acids, there were indications of cross resistance developing.

It is known that cellular proteins are often involved in resistance development against numerous antimicrobial agents, and more specifically the outer membrane of Gram-negative bacteria. *Listeria* is a Gram-positive bacterium and the involvement of proteins may be more complicated. Current results have, however, provided some valuable information on which to build further in-depth investigations. It may even be possible to use the results in the prediction of resistance developing against antibiotics as well as food preservatives.

4.5 REFERENCES

Jay, J.M. 2000. Modern food microbiology, 6th ed Aspen Publishers, Inc., Gaithersburg, Md.

Perreten, V., Schwarz, F., Cresta, L., Boeglin, M., Dasen, G. And Teuber, M. 1997. Antibiotic resistance spread in food. *Nature,* 380:801-802.

Poros-Gluchowska, J. and Markiewicz, Z. 2003. Antimicrobial resistance of *Listeria monocytogenes. Acta Microbiologica Polonica*, 52:113-129.

Poyart-Salmeron, C., Carlier, C., Trieu-Cuot, P., Courtieu, A.L. and Courvalin, P. 1990. Transferable plasmid-mediated antibiotic resistance in *Listeria monocytogenes*. *The Lancet,* 335:1422-1426.

Rocourt, J. and Bille, J. 1997. Foodborne listeriosis. World Health Stat. Q. 50 (1-2), 67-73.

Teuber, M. 1999. Spread of antibiotic resistance with foodborne pathogens, CMSL Cell Molecular Life Science, 56:755-761.

Theron, M.M. and Lues, J.F.R. 2007. Organic acids and meat preservation: a review. *Food Reviews International*, 23:141-158.

CHAPTER 5

GENERAL DISCUSSION

5.1 BACKGROUND

Listeria is a food-borne pathogen commonly found in the environment. As such, the organism is often consumed in small amounts by the general public without apparent ill effects as it is believed that only higher levels of *Listeria* will cause severe problems. Although the genus currently consists of six species and several of these species have been implicated in human infections *L. monocytogenes* is the only species recognised as a human pathogen.

The bacterium later named *Listeria monocytogenes* was first isolated from diseased laboratory animals and a description published in 1926 (Murray et al., 1926). This publication did not result in immediate interest and research consisted largely of reports of different animals and birds that suffered from listeriosis. Reports on transmission of the disease to humans were increasing and during 1940 to 1960, research efforts were established by Gray (1963). This led to isolation methods and increasing knowledge on the widespread of this organism in the environment (Gray, 1963). In the late 1940s there was some evidence in Germany of the possible role of food in human listeriosis but no research on this potential cause of food-borne illness was performed. However, in the 1980s this changed after three major outbreaks of listeriosis in North America were attributed to contaminated food.

5.2 ISOLATION AND IDENTIFICATION OF *LISTERIA*

Isolation of *Listeria* from food samples has been reported to be problematic due to small numbers of the organism often being suppressed by large quantities of normal habitants. Enrichment methods have been developed and were used in the current study. As such a wide range of *Listeria* spp. could be isolated. Routine identification procedures, which include Gram-staining, catalase reaction, motility and biochemical identification (API *Listeria*) were found adequate to identify the isolates to genus level. However, reliable species identification could only be achieved by 16S rDNA sequencing. From 200 samples 51 were identified as *Listeria* spp. by the API *Listeria* kit as well as with 16S rDNA. However, species identification was more accurately confirmed by the DNA based method (Chapter 2, Table 2.2).

5.3 ANTIMICROBIAL SUSCEPTIBILITY OF *LISTERIA*

Susceptibility of *Listeria* spp. isolated from various food premises was determined against a wide range of antimicrobial agents, including antibiotics used in current treatment regimes for *Listeria* infections and organic acids commonly used in food preservation (Chapter 3). High level antibiotic susceptibility was observed in the majority of the isolates, except for individual resistance in one *L. innocua* strain, which showed exceptionally high MIC values for amikacin, gentamicin and trimethoprim-sulfamethoxazole. However this strain was much more susceptible to levofloxacin than

any of the other isolates. Although all the *L. monocytogenes* isolates were susceptible for imipenem, the control *L. monocytogenes* ATCC 19111 showed high level resistance. Treatment of choice for *Listeria* infections includes ampicillin or penicillin G in combination with an aminoglycoside, such as gentamicin. It was interesting to note that in the current study, reduced susceptibility in *L. monocytogenes* was found only against gentamicin and clindamycin. Tetracycline resistance has been reported to be the most frequently resistance encountered in *L. monocytogenes* isolated from humans and also increasingly found in *Listeria* spp. isolated from food and environmental sources. In the current study the MIC levels recorded for tetracycline were notably low.

Decreased organic acid activity was evident at the higher pH especially for the salts potassium sorbate, sodium benzoate and sodium propionate. All the isolates and control strains were resistant to sodium citrate at pH 5 as well as pH 7. At a more acidic pH value organic acids are found in their un-dissociated form and as such they can freely diffuse across the bacterial cell membrane into the cell. Organic acids should, therefore, be more inhibitory in acidic foodstuff. However, *Listeria* is notorious of growing at extremely low pH values and susceptibility against these food preservatives should be mandatory.

An indirect relationship between the susceptibility to the organic acids and antibiotics was found in isolated cases, where an organism showed high minimum inhibitory concentration (MIC) values for various antibiotics, but at the same time was highly sensitive for all the organic acids. However, the majority of the isolates did not follow a

similar trend and in depth studies of the mechanisms involved in resistance development may reveal valuable information with regard to successful application of antibiotics as well as the organic acids in clinical therapy and food preservation.

5.4 INDUCING ANTIMICROBIAL RESISTANCE

Susceptible isolates and control strains were exposed to increasing concentrations of antibiotics and organic acids in attempting to simulate resistance development. Any change in susceptibility was monitored by comparing MICs before and after induction. Cellular proteins are often involved in resistance development and isolates that survived at various exposing concentrations were selected to investigate the possible alterations in cellular protein profiles (Chapter 4).

Increased MIC values were recorded after exposure, although this induction did not result in high level resistance against all the antimicrobial agents. Overall increased MIC values were demonstrated in tetracycline and ciprofloxacin after induction with the respective agents, while exposure to some organic acids resulted in notable increases in MIC values of antibiotics such as ampicillin and ciprofloxacin in some *L. monocytogenes* isolates. On the other hand induction with tetracycline demonstrated increased MIC values of acetic acid also in *L. monocytogenes*.

Protein bands visible in all the induced strains were distributed over a wide range of protein sizes. No obvious correlation could be demonstrated by visual observation of resulting protein profiles. Overall, the induced strains produced more protein bands, in particular induction with tetracycline, while only a few well defined protein bands could be clearly detected in all the isolates. It would be necessary to conduct a comparison of total protein profiles to demonstrate the differences between species and serovars in order to identify specific alterations in protein composition as a result of decreased susceptibility.

Evidence was provided that continuous exposure to antimicrobial agents may cause *Listeria* spp. to develop resistance to different antimicrobial agents. Although concurrent resistance development against antibiotics as well as organic acids was not unequivocally demonstrated, there were indications of cross resistance developing. Any indications of cross resistance development may play a significant role in clinical treatment of *Listeria* infections, as well as effective food preservation.

5.5. FUTURE RESEARCH

The study has revealed various factors associated with the problems encountered in the control of *Listeria* in the food matrix and has opened up numerous research opportunities:

- Continuous monitoring of the prevalence of *Listeria* spp. in South African food production.
- Correlate prevalence of *Listeria* in food with possible listeriosis and isolation from clinical samples.
- Transmission of resistance between food isolates and *Listeria* from clinical infections.
- In depth studies on mechanisms involved in the development of resistance to food preservatives, such as the presence of plasmids and virulence genes.

5.6. REFERENCES

Gray, M.L. 1963. Epidemiological aspects of listeriosis. *American Journal of Public Health*, 53:554-563.

Murray, E.G.D., Webb, R.A. and Swann, M.B.R. 1926. A disease of rabbit characterised by a large mononuclear leucocytosis, caused by a hitherto undescribed bacillus *Bacterium monocytogenes (*n. sp.). *Journal of Pathology Bacteriology,* 29:407-439.

APPENDIX A

Table A.1: Antibiotic susceptibility of *Listeria innocua* isolates (n = 8).

		MIC (µg/ml)										
Sample no	**Origin***	**AMK**	**AMP**	**CIP**	**CD**	**ERY**	**GEN**	**IMI**	**LEV**	**PEN**	**TET**	**TS**
84	CA head and claws	8	2	1	0.75	≤0.25	2	2	2	≤0.25	0.5	0.047
105	RTE tuna pasta	4	1	1	0.75	≤0.25	2	0.75	1	≤0.25	1	0.047
151	DAIRY raw milk	8	1	1	0.5	≤0.25	2	0.75	2	≤0.25	1	0.047
152	DAIRY raw milk	8	1	1	0.5	≤0.25	2	0.75	2	≤0.25	1	0.125
153	DAIRY raw milk	4	1	1	0.75	≤0.25	2	1.5	2	≤0.25	1	0.125
157	DAIRY raw yoghurt	8	1	2	0.75	≤0.25	1	>32	2	≤0.25	1	>32
158	DAIRY raw yoghurt	8	1	2	0.75	≤0.25	4	>32	2	≤0.25	1	>32
172	DAIRY raw milk	>256	4	1	0.75	≤0.25	64	>32	≤0.25	≤0.25	1	>32

*CA = chicken abattoir, RTE = ready-to-eat.

AMK = amikacin, AMP = ampicillin, CIP = ciprofloxacin, CD = clindamycin, ERY = erythromycin, GEN = gentamicin, IMI = imipenem, LEV = levofloxacin, PEN = penicillin, TET = tetracycline, TS = trimethoprim/sulphamethoxazole

Table A.2: Antibiotic susceptibility of *Listeria monocytogenes* isolates (n = 6).

		MIC (µg/ml)										
Sample no	**Origin***	**AMK**	**AMP**	**CIP**	**CD**	**ERY**	**GEN**	**IMI**	**LEV**	**PEN**	**TET**	**TS**
60	CA offal (neck, intestines)	8	2	0.5	0.75	≤0.25	1	0.5	1	≤0.25	0.5	0.032
77	CA water after 1st rinsing	8	2	0.5	0.5	≤0.25	2	0.75	2	≤0.25	≤0.25	0.032
126	RTE sashimi	8	1	0.5	0.25	≤0.25	4	1	1	≤0.25	1	0.047
138	RTE california roll (prawn)	8	1	1	12	≤0.25	1	0.75	1	≤0.25	1	0.047
139	RTE maki	4	1	1	0.25	≤0.25	1	0.75	1	≤0.25	1	0.094
141	RTE california roll (salmon)	4	0.5	0.5	0.27	≤0.25	1	0.5	1	≤0.25	1	0.047

*CA = chicken abattoir, RTE = ready-to-eat.

AMK = amikacin, AMP = ampicillin, CIP = ciprofloxacin, CD = clindamycin, ERY = erythromycin, GEN = gentamicin, IMI = imipenem, LEV = levofloxacin, PEN = penicillin, TET = tetracycline, TS = trimethoprim/sulphamethoxazole

Table A.3: Antibiotic susceptibility of *Listeria welshimeri* isolates (n = 36).

		MIC (µg/ml)										
Sample no	**Origin***	**AMK**	**AMP**	**CIP**	**CD**	**ERY**	**GEN**	**IMI**	**LEV**	**PEN**	**TET**	**TS**
51	CA piece of meat on floor	4	1	1	0.5	≤0.25	1	0.75	1	≤0.25	0.5	0.094
53	CA water after de-feathering	8	2	0.5	0.5	≤0.25	2	0.38	1	≤0.25	0.5	0.094
54	CA brine water	8	1	0.5	0.5	≤0.25	2	0.5	1	≤0.25	0.5	0.094
55	CA drumstick	8	0.5	0.5	0.5	≤0.25	1	0.25	1	≤0.25	0.5	0.094
56	CA wing	8	0.5	0.5	0.5	≤0.25	1	0.25	1	≤0.25	0.5	0.094
57	CA small fillet	4	0.5	0.5	0.5	≤0.25	1	0.38	1	≤0.25	0.5	0.094
58	CA large fillet	8	0.5	0.5	0.5	≤0.25	2	0.5	2	≤0.25	0.5	0.094
59	CA bone after deboning	8	0.5	0.5	0.75	≤0.25	2	0.38	2	≤0.25	0.5	0.094
61	CA frozen piece of meat	8	0.5	0.5	0.5	≤0.25	1	0.38	1	≤0.25	0.5	0.032
62	CA ice from frozen meat	4	2	0.5	0.25	≤0.25	2	0.5	2	≤0.25	0.5	0.047
63	CA frozen piece of meat	4	1	0.5	0.5	≤0.25	2	0.25	2	≤0.25	0.5	0.064
64	CA ice scraping	8	0.5	0.5	1	≤0.25	2	0.75	2	≤0.25	0.5	0.047
65	CA whole carcass	8	2	0.5	0.75	≤0.25	2	0.75	1	≤0.25	0.5	0.016
66	CA stomach	4	0.5	0.5	0.5	≤0.25	1	0.5	1	≤0.25	0.5	0.016
67	CA intestines	4	1	0.5	0.5	≤0.25	1	0.38	2	≤0.25	0.5	0.047
75	CA feathers	4	0.5	0.5	0.5	≤0.25	1	0.25	1	≤0.25	0.5	0.032
76	CA drain sample	8	2	0.5	0.5	≤0.25	2	0.5	4	≤0.25	0.5	0.032
79	CA water after de-feathering	8	2	0.5	0.5	≤0.25	2	0.5	2	≤0.25	0.5	0.032
80	CA brine water	8	0.5	0.5	0.5	≤0.25	1	0.5	1	≤0.25	0.5	0.064
81	CA drain sample	8	0.5	0.5	0.5	≤0.25	1	0.5	1	≤0.25	0.5	0.047
82	CA feathers	8	0.5	0.5	0.38	≤0.25	2	0.38	2	≤0.25	0.5	0.047
83	CA intestines	8	2	1	0.75	≤0.25	2	0.5	2	≤0.25	0.5	0.047
85	CA intestines	4	2	0.5	0.75	≤0.25	1	1.5	2	≤0.25	0.5	0.047
86	CA wing	8	2	0.5	0.75	≤0.25	1	1	1	≤0.25	0.5	0.047
87	CA liver	8	2	0.5	0.5	≤0.25	1	0.5	2	≤0.25	0.5	0.023
88	CA stomach	8	2	0.5	0.75	≤0.25	1	0.75	2	≤0.25	0.5	0.094
89	CA stomach	8	2	1	0.75	≤0.25	1	1	2	≤0.25	0.5	0.064
91	CA neck	8	2	0.5	0.75	≤0.25	2	0.75	1	≤0.25	0.5	0.064
92	CA drumstick after brine dip	8	2	0.5	0.75	≤0.25	2	0.75	2	≤0.25	0.5	0.064
95	CA fresh piece before packaging	8	0.5	0.5	0.75	≤0.25	1	0.75	1	≤0.25	0.5	0.032
96	CA bone after deboning	8	2	0.5	0.75	≤0.25	2	0.75	2	≤0.25	1	0.032
97	CA fillet	4	0.5	0.5	0.75	≤0.25	1	0.5	1	≤0.25	1	0.047
98	CA fillet	4	0.5	0.5	0.75	≤0.25	1	0.25	1	≤0.25	1	0.047
99	CA ice scrapings	4	0.5	0.5	0.75	≤0.25	1	0.5	1	≤0.25	1	0.032
115	RTE Pasta salad	8	0.5	0.5	0.75	≤0.25	1	0.25	1	≤0.25	1	0.047

*CA = chicken abattoir, RTE = ready-to-eat

Table A.4: Susceptibility of *Listeria monocytogenes* isolates to organic acids (n = 6) at pH 5.

		MIC (mM)												
Sample no	**Origin***	**ACE**	**BNZ**	**BUT**	**CIT**	**FMC**	**LAC**	**MAL**	**PSB**	**PPN**	**SBZ**	**SCT**	**SPP**	**SOR**
60	CA offal (neck, intestines)	2.5	5	5	2.5	2.5	10	10	20	2.5	20	>150	20	10
77	CA water after 1st rinsing	5	10	10	2.5	2.5	10	10	20	2.5	20	100	20	10
126	RTE sashimi	5	2.5	10	2.5	2.5	10	5	50	5	5	>150	150	10
138	RTE californian roll (prawn)	2.5	1	10	2.5	2.5	10	5	10	2.5	5	>150	10	5
139	RTE maki	2.5	1	5	2.5	2.5	10	2.5	10	2.5	5	>150	5	5
141	RTE californian roll (salmon)	5	1	10	2.5	2.5	10	5	10	2.5	5	>150	10	5

*CA = chicken abattoir, RTE = ready-to-eat.

ACE = acetic acid, BNZ = benzoic acid, BUT = butyric acid, CIT = citric acid, FMC = fumaric acid, MAL = malic acid, PSB = potassium sorbate, SCT = sodium citrate, SPP = sodium propionate, SOR = sorbic acid.

Table A.5: Susceptibility of *Listeria monocytogenes* isolates to organic acids (n = 6) at pH 7.

		MIC (mM)												
Sample no	**Origin***	**ACE**	**BNZ**	**BUT**	**CIT**	**FMC**	**LAC**	**MAL**	**PSB**	**PPN**	**SBZ**	**SCT**	**SPP**	**SOR**
60	CA offal (neck, intestines)	25	30	50	20	20	50	20	200	25	150	>150	300	20
77	CA water after 1st rinsing	50	30	50	20	20	50	20	200	25	150	>150	300	20
126	RTE sashimi	50	20	25	20	25	50	20	300	50	150	>150	300	>30
138	RTE californian roll (prawn)	25	20	25	20	25	100	20	300	50	150	>150	300	>30
139	RTE maki	25	20	25	20	25	50	20	300	50	150	>150	250	>30
141	RTE californian roll (salmon)	25	20	25	20	25	50	20	300	25	150	>150	300	>30

*CA = chicken abattoir, RTE = ready-to-eat.

ACE = acetic acid, BNZ = benzoic acid, BUT = butyric acid, CIT = citric acid, FMC = fumaric acid, MAL = malic acid, PSB = potassium sorbate, SCT = sodium citrate, SPP = sodium propionate, SOR = sorbic acid.

Table A.6: Susceptibility of *Listeria welshimeri* isolates to organic acids (n = 36) at pH 5.

		MIC (mM)												
Sample no	**Origin***	**ACE**	**BNZ**	**BUT**	**CIT**	**FMC**	**LAC**	**MAL**	**PSB**	**PPN**	**SBZ**	**SCT**	**SPP**	**SOR**
51	CA piece of meat on floor	2.5	5	10	2.5	2.5	10	10	20	2.5	20	>150	20	5
53	CA water after defeathering	5	5	10	2.5	2.5	10	10	20	2.5	20	>150	20	10
54	CA brine water	5	5	10	2.5	2.5	10	10	20	2.5	20	>150	20	10
55	CA drumstick	2.5	5	5	2.5	2.5	10	10	20	2.5	20	>150	20	10
56	CA wing	5	5	10	2.5	2.5	10	10	20	2.5	20	>150	20	10
57	CA small fillet	5	5	10	2.5	2.5	10	10	20	2.5	20	>150	20	10
58	CA large fillet	5	5	10	2.5	2.5	10	10	20	2.5	20	>150	20	10
59	CA bone after deboning	5	5	10	2.5	2.5	10	10	20	2.5	20	>150	20	10
61	CA frozen piece of meat	5	5	10	2.5	2.5	10	10	20	2.5	20	>150	20	10
62	CA ice from frozen meat	5	10	10	2.5	2.5	10	10	20	2.5	20	>150	20	10
63	CA frozen piece of meat	5	10	10	2.5	2.5	10	10	20	2.5	20	>150	20	10
64	CA ice scraping	5	10	10	2.5	2.5	10	10	20	2.5	20	>150	20	10
65	CA whole carcass	2.5	10	10	2.5	2.5	10	10	20	2.5	20	>150	20	10
66	CA stomach	2.5	5	5	2.5	2.5	10	10	20	2.5	20	>150	20	10
67	CA intestines	5	10	10	2.5	2.5	10	10	20	2.5	20	>150	20	10
75	CA feathers	5	10	10	2.5	2.5	10	10	20	2.5	20	>150	20	10
76	CA drain sample	5	10	10	2.5	2.5	5	10	20	2.5	20	>150	20	10
79	CA water after defeathering	2.5	10	5	2.5	2.5	10	10	20	2.5	20	>150	20	10
80	CA brine water	2.5	10	5	2.5	2.5	10	10	20	2.5	20	>150	20	10
81	CA drain sample	5	5	5	2.5	2.5	10	10	20	2.5	20	>150	20	10
82	CA feathers	5	10	5	2.5	5	10	10	20	2.5	20	>150	20	10
83	CA intestines	5	10	10	2.5	2.5	10	10	20	2.5	20	>150	20	10
85	CA intestines	2.5	10	5	2.5	2.5	10	10	20	2.5	20	>150	50	10
86	CA wing	5	5	5	2.5	2.5	10	10	20	2.5	20	>150	10	10
87	CA liver	≤0.5	1	5	2.5	2.5	10	2.5	10	2.5	≤0.5	>150	5	5
88	CA stomach	≤0.5	1	5	2.5	2.5	10	2.5	10	2.5	≤0.5	>150	5	5
89	CA stomach	≤0.5	1	5	2.5	2.5	10	2.5	10	2.5	1	>150	5	5
91	CA neck	≤0.5	1	5	2.5	2.5	10	2.5	5	2.5	1	>150	5	5
92	CA drumstick after brine dip	≤0.5	1	5	2.5	2.5	10	2.5	2.5	2.5	≤0.5	>150	5	5
95	CA fresh piece before packaging	≤0.5	1	5	2.5	2.5	10	2.5	10	2.5	≤0.5	>150	5	5
96	CA bone after deboning	1	1	5	2.5	2.5	10	2.5	10	2.5	1	>150	5	5
97	CA fillet	2.5	1	5	2.5	2.5	10	2.5	10	2.5	1	>150	5	5
98	CA fillet	2.5	1	10	2.5	2.5	10	2.5	10	2.5	≤0.5	>150	5	5
99	CA Ice scrapings	5	2.5	10	2.5	2.5	10	5	10	2.5	10	>150	10	10
115	RTE Pasta salad	1	1	10	2.5	2.5	10	5	10	2.5	5	>150	10	5

*CA = chicken abattoir, RTE = ready-to-eat.

ACE = acetic acid, BNZ = benzoic acid, BUT = butyric acid, CIT = citric acid, FMC = fumaric acid, MAL = malic acid, PSB = potassium sorbate, SCT = sodium citrate, SPP = sodium propionate, SOR = sorbic acid.

Table A.7: Susceptibility of *Listeria welshimeri* isolates to organic acids (n = 36) at pH 7.

		MIC (mM)												
Sample no	**Origin***	**ACE**	**BNZ**	**BUT**	**CIT**	**FMC**	**LAC**	**MAL**	**PSB**	**PPN**	**SBZ**	**SCT**	**SPP**	**SOR**
51	CA piece of meat on floor	25	20	50	20	20	50	20	200	25	150	>150	300	20
53	CA water after defeathering	25	30	50	20	20	50	20	200	25	150	>150	300	20
54	CA brine water	25	30	50	20	20	50	20	200	25	150	>150	300	20
55	CA drumstick	25	30	20	20	20	50	20	200	25	150	>150	300	20
56	CA wing	25	30	50	20	20	50	20	200	25	150	>150	300	20
57	CA small fillet	25	30	50	20	20	50	20	200	25	150	>150	300	20
58	CA large fillet	25	30	50	20	20	50	20	200	25	150	>150	300	20
59	CA bone after deboning	25	30	50	20	20	50	20	200	25	150	>150	300	20
61	CA frozen piece of meat	25	30	50	20	20	50	20	200	25	150	>150	300	20
62	CA ice from frozen meat	25	30	50	20	20	50	20	200	25	150	>150	300	20
63	CA frozen piece of meat	25	30	50	20	20	50	20	200	25	150	>150	300	20
64	CA ice scraping	25	30	50	20	20	50	20	200	25	150	>150	300	20
65	CA whole carcass	25	30	50	20	20	50	20	200	25	150	>150	300	20
66	CA stomach	25	30	50	20	20	50	20	200	25	150	>150	300	20
67	CA intestines	25	30	50	20	20	50	20	200	25	150	>150	300	20
75	CA feathers	50	30	50	20	20	50	20	200	25	150	>150	300	20
76	CA drain sample	50	30	50	20	20	50	20	200	25	150	>150	300	20
79	CA water after defeathering	50	30	50	20	20	50	20	200	25	150	>150	300	20
80	CA brine water	50	30	50	20	20	50	20	200	25	150	>150	300	20
81	CA drain sample	25	30	20	20	20	50	20	200	25	150	>150	300	20
82	CA feathers	50	30	50	20	20	50	20	200	25	150	>150	300	20
83	CA intestines	50	30	50	20	20	50	20	200	25	150	>150	300	20
85	CA intestines	50	30	50	20	20	50	20	200	25	150	>150	300	20
86	CA wing	25	30	50	20	20	50	20	200	25	150	>150	300	20
87	CA liver	20	20	25	20	20	50	20	300	25	100	150	150	>30
88	CA stomach	20	20	25	20	20	50	20	300	25	100	150	150	>30
89	CA stomach	20	20	25	20	20	50	20	300	25	100	150	150	>30
91	CA neck	20	20	25	20	20	50	20	300	25	100	150	150	>30
92	CA drumstick after brine dip	20	20	25	20	20	50	20	300	25	100	>150	150	>30
95	CA fresh piece before packaging	20	20	25	20	25	50	20	300	25	100	150	150	>30
96	CA bone after deboning	20	20	25	20	25	50	20	300	25	100	150	150	>30
97	CA fillet	20	20	25	20	25	50	20	300	25	100	150	150	>30
98	CA fillet	25	20	25	20	25	50	20	300	25	150	>150	150	>30
99	CA Ice scrapings	50	20	25	20	25	50	20	300	25	150	>150	150	>30
115	RTE Pasta salad	25	20	25	20	25	50	20	300	25	150	>150	150	>30

*CA = chicken abattoir, RTE = ready-to-eat.

Table A.8: Susceptibility of *Listeria innocua* isolates to organic acids (n = 8) at pH 5.

		MIC (mM)												
Sample no	**Origin***	**ACE**	**BNZ**	**BUT**	**CIT**	**FMC**	**LAC**	**MAL**	**PSB**	**PPN**	**SBZ**	**SCT**	**SPP**	**SOR**
84	CA head and claws	25	10	10	2.5	5	10	10	20	2.5	20	>150	20	10
105	RTE tuna pasta	1	1	5	2.5	2.5	10	5	10	5	5	>150	5	5
151	DAIRY raw milk	5	1	10	2.5	2.5	10	5	10	5	5	>150	10	5
152	DAIRY raw milk	5	1	10	2.5	2.5	10	5	10	5	5	>150	10	5
153	DAIRY raw milk	5	1	10	2.5	2.5	10	5	50	10	5	>150	10	5
157	DAIRY raw yoghurt	25	1	10	5	2.5	10	5	10	10	5	>150	10	5
158	DAIRY raw yoghurt	25	1	10	5	2.5	10	5	10	10	5	>150	10	5
172	DAIRY raw milk	2.5	1	10	5	2.5	10	5	10	5	5	>150	10	5

*CA = chicken abattoir, RTE = ready-to-eat.

ACE = acetic acid, BNZ = benzoic acid, BUT = butyric acid, CIT = citric acid, FMC = fumaric acid, MAL = malic acid, PSB = potassium sorbate, SCT = sodium citrate, SPP = sodium propionate, SOR = sorbic acid.

Table A.9: Susceptibility of *Listeria innocua* isolates to organic acids (n = 8) at pH 7.

		MIC (mM)												
Sample no	**Origin***	**ACE**	**BNZ**	**BUT**	**CIT**	**FMC**	**LAC**	**MAL**	**PSB**	**PPN**	**SBZ**	**SCT**	**SPP**	**SOR**
84	CA head and claws	50	30	50	20	20	50	20	200	25	150	>150	300	20
105	RTE tuna pasta	50	20	25	20	25	50	20	300	50	150	>150	150	>30
151	DAIRY raw milk	50	20	25	20	25	50	20	300	50	150	>150	300	>30
152	DAIRY raw milk	50	20	25	20	25	50	20	300	50	150	>150	300	>30
153	DAIRY raw milk	50	20	25	20	25	50	20	300	50	150	>150	300	>30
157	DAIRY raw yoghurt	25	20	25	25	25	50	20	300	50	150	>150	300	>30
158	DAIRY raw yoghurt	25	20	25	25	25	50	20	150	50	150	>150	250	>30
172	DAIRY raw milk	25	20	25	20	25	50	20	150	50	150	>150	250	>30

*CA = chicken abattoir, RTE = ready-to-eat.

ACE = acetic acid, BNZ = benzoic acid, BUT = butyric acid, CIT = citric acid, FMC = fumaric acid, MAL = malic acid, PSB = potassium sorbate, SCT = sodium citrate, SPP = sodium propionate, SOR = sorbic acid.

APPENDIX B

Table B.1: Total protein profiles of *Listeria monocytogenes* 60 after induction with various antimicrobial agents. Notable MIC increases are depicted below the protein bands.

	Induced strain[ab]					Visible protein bands (kDa)
	6O no induction	60 CA	60 LA	60 CP	60 TE	
				+		9.8
	+	+	+			32.5
	+	+	+			33.3
	+	+	+			33.6
	+			+	+	37.2
				+	+	41.4
				+	+	44.5
					+	47
				+		48.5
				+		85.1
				+		104.3
Ciprofloxacin	0.5 µg/ml			128 µg/ml		
Tetracycline	0.5 µg/ml				16 µg/ml	

a Inducing agent: CA = citric acid, LA = lactic acid, CP = ciprofloxacin, TE = tetracycline.
b Final induction concentrations: citric acid 20 mM, lactic acid 40 mM, ciprofloxacin 32 µg/ml, tetracycline 32 µg/ml.

Table B.2: Total protein profiles of *Listeria monocytogenes* 138 after induction with various antimicrobial agents. Notable MIC increases are depicted below the protein bands.

	Induced strain[ab]				Visible protein bands (kDa)
	138 no induction	138 AA	138 LA	138 TE	
				+	5.4-27.5
	+			+	32.7
		+		+	33.3
	+			+	33.6
	+			+	35.3
	+		+		37
	+				37.2
				+	39.6
				+	41.4
				+	44.5-113.8
Ampicillin	1 µg/ml	4 µg/ml		4 µg/ml	
Tetracycline	1 µg/ml			16 µg/ml	

a Inducing agent: AA = Acetic acid, LA = lactic acid, TE = tetracycline.
b Final induction concentrations: acetic acid 40 mM, lactic acid 40 mM, tetracycline 32 µg/ml

Table B.3: Total protein profiles of *Listeria monocytogenes* 139 after induction with various antimicrobial agents. Notable MIC increases are depicted below the protein bands.

	Induced strain[ab]				Visible protein bands (kDa)
	139 no induction	139 AA	139 CA	139 TE	
				+	5.4
				+	9.8
	+				11.6
				+	15.8-32
		+	+		33.3
	+	+	+		33.6
		+	+		35.3
				+	39.6
				+	41.4
			+		47
				+	48.5
				+	50.3
			+		54.4
				+	57.7-80.6
		+		+	85.1
			+		87.9
				+	89-135.2
Ampicillin	1 µg/ml	4 µg/ml	4 µg/ml	4 µg/ml	
Tetracycline	1 µg/ml			16 µg/ml	
Acetic acid	2.5 mM	10 mM	10 mM	10 mM	

a Inducing agent: AA = Acetic acid, CA = citric acid, TE = tetracycline.
b Final induction concentrations: acetic acid 40 mM, citric acid 20 mM, tetracycline 32 µg/ml

Table B.4: Total protein profiles of *Listeria monocytogenes* ATCC 19111 after induction with various antimicrobial agents. Notable MIC increases are depicted below the protein bands.

	Induced strain[ab]			Visible protein bands (kDa)
	No induction	**AA**	**LA**	
	+			5.4
			+	9.8
	+	+	+	13.2
	+	+	+	17.5
	+	+	+	20.3
	+	+	+	32.7
		+		33.3
	+	+	+	33.6
	+			37
	+			37.2
			+	39.6
		+	+	44.5
			+	51.6
	+	+	+	59.8
			+	61.5
		+		63.6
			+	69.5
		+		71.1
		+	+	75.3
		+		78
	+	+	+	85.1
	+	+	+	95.5
Ciprofloxacin	0.5 µg/ml	2 µg/ml	2 µg/ml	

a Inducing agent: AA = Acetic acid, LA = lactic acid.
b Final induction concentrations: acetic acid 40 mM, lactic acid 40 mM.

Table B.5: Total protein profiles of *Listeria welshimeri* 87 after induction with various antimicrobial agents. Notable MIC increases are depicted below the protein bands.

	Induced strain[ab]					Visible protein bands (kDa)
	87 no induction	87 LA	87 PE	87 CP	87 TE	
			+		+	7.4
		+		+		9.8
		+	+			17.5
			+	+		27.5
	+		+			32
	+	+	+	+		32.7
	+	+	+		+	33.6
		+	+	+		35.3
			+			37
				+	+	37.3
				+	+	39.6
			+		+	41.4
						44.5
				+	+	48.5
			+			50.3
						51.6
			+	+		54.4
			+	+		57.7
	+		+			63.6
			+		+	69.5
			+	+		80.6
		+	+	+		87.9
						89
			+		+	93.4
			+			95.5
			+			100.8
			+			104.3
			+	+		107.8
				+		113.8
			+			122
Ampicillin	2 µg/ml		64 µg/ml			
Ciprofloxacin	0.5 µg/ml			128 µg/ml		
Penicillin	≤0.25 µg/ml		32 µg/ml			
Tetracycline	0.5 µg/ml			2 µg/ml	16 µg/ml	
Acetic acid	≤0.5 mM	5 mM	5mM	5 mM		

a Inducing agent: LA = lactic acid, PE = Penicillin, CP = ciprofloxacin, TE = tetracycline.
b Final induction concentrations: lactic acid 40 mM, penicillin 1 µg/ml, ciprofloxacin 32 µg/ml, tetracycline 32 µg/ml.

Table B.6: Total protein profiles of *Listeria welshimeri* 88 after induction with various antimicrobial agents. Notable MIC increases are depicted below the protein bands.

	Induced strain[ab]					Visible protein bands (kDa)
	88 no induction	88 CA	88 LA	88 CP	88 TE	
				+		9.8
	+	+	+			32.7
		+				33.3
		+		+		33.6
		+				35.3
		+				37.3
				+	+	39.3
				+		41.4
				+		44.5
				+		48.5
				+		82.3
				+	+	104.3
Ciprofloxacin	0.5 µg/ml			128 µg/ml		
Tetracycline	0.5 µg/ml				16 µg/ml	

a Inducing agent: CA = citric acid, LA = lactic acid, CP = ciprofloxacin, TE = tetracycline.
b Final induction concentrations: citric acid 20 mM, lactic acid 40 mM, ciprofloxacin 32 µg/ml, tetracycline 32 µg/ml.

Table B.7: Total protein profiles of *Listeria welshimeri* 92 after induction with various antimicrobial agents. Notable MIC increases are depicted below the protein bands.

	Induced strain[ab]				Visible protein bands (kDa)
	92 no induction	92 CA	92 CP	92 TE	
	+	+	+		5.4
	+	+			15.8
	+	+			17.5
	+	+			23.2
			+	+	35.3
			+	+	37.3
	+				39.6
	+	+	+		41.4
			+		44.5
	+	+	+	+	47
	+	+		+	48.5
			+		54.4
		+			63.6
	+	+			71.1
		+			72.6
				+	75.3
			+		78
		+		+	82.3
	+		+		85.1
		+			87.9
		+			93.4
			+		98.6
				+	100.8
Ciprofloxacin	0.5 μg/ml		128 μg/ml		
Tetracycline	0.5 μg/ml			16 μg/ml	
Acetic acid	≤0.5 mM	10 mM			

a Inducing agent: CA = citric acid, CP = ciprofloxacin, TE = tetracycline.
b Final induction concentrations: citric acid 20 mM, ciprofloxacin 32 μg/ml, tetracycline 32 μg/ml.

Table B.8: Total protein profiles of *Listeria welshimeri* 95 after induction with various antimicrobial agents. Notable MIC increases are depicted below the protein bands.

	Induced strain[ab]				Visible protein bands (kDa)
	95 no induction	95 LA	95 CP	95 TE	
	+				5.4
			+	+	7.4
	+	+			15.8
		+			17.5
	+	+			20.3
	+	+			23.2
			+	+	35.3
			+	+	39.6
	+	+		+	41.4
				+	44.5
			+	+	47.1
	+	+			48.5
			+	+	72.6
			+		82.3
				+	85.1
	+				87.9
		+			89
			+		100.8
				+	104.3
Ciprofloxacin	0.5 µg/ml		128 µg/ml		
Tetracycline	0.5 µg/ml			16 µg/ml	
Acetic acid	≤0.5 mM	5 mM	5 mM	5 mM	

a Inducing agent: LA = Lactic acid, CP = ciprofloxacin, TE = tetracycline.
b Final induction concentrations: lactic acid 40 mM, ciprofloxacin 32 µg/ml, tetracycline 32 µg/ml.

Table B.9: Total protein profiles of *Listeria welshimeri* ATCC 35897 after induction with various antimicrobial agents. Notable MIC increases are depicted below the protein bands.

	Induced strain[ab]			Visible protein bands (kDa)
	No induction	CA	TE	
			+	5.4
			+	13.2
				20.3
		+		23.2
	+			32.3
	+	+		33.6
	+	+		35.3
			+	41.4
	+			44.5
			+	48.5
			+	69.5
	+			78
	+		+	85.1
Tetracycline	≤0.25 µg/ml		8 µg/ml	
Acetic acid	1 mM	10 mM	10 mM	

a Inducing agent: CA = Citric acid, TE = tetracycline.
b Final induction concentrations: citric acid 20 mM, tetracycline 2 µg/ml.

Table B.10: Total protein profiles of *Listeria innocua* 105 after induction with various antimicrobial agents. Notable MIC increases are depicted below the protein bands.

	Induced strain[ab]					Visible protein bands (kDa)
	105 no induction	105 AA	105 CA	105 LA	105 TE	
					+	7.4
	+					9.8
	+					11.6
	+					23.2
	+	+	+			32.5
	+					32.7
	+	+				33.3
	+	+				33.6
	+	+	+			35.3
	+	+				37
	+	+	+	+		37.3
					+	39.6
					+	47.1
					+	63.6
					+	69.5
					+	80.6
					+	82.3
					+	93.4
					+	98.6
					+	104.3
Ampicillin	1 µg/ml	4 µg/ml	4 µg/ml		4 µg/ml	
Tetracycline	1 µg/ml				16 µg/ml	

a Inducing agent: AA = acetic acid, CA = citric acid, LA = lactic acid, TE = tetracycline.
b Final induction concentrations: acetic acid 40 mM, citric acid 20 mM, lactic acid 40 mM, tetracycline 32 µg/ml.

Table B.11: Total protein profiles of *Listeria innocua* ATCC 33090 after induction with various antimicrobial agents. Notable MIC increases are depicted below the protein bands.

	Induced strain[ab]					Visible protein bands (kDa)
	No induction	AA	CA	LA	TE	
					+	5.4
	+	+	+	+		7.4
				+		11.6
	+		+	+		13.2
					+	15.8
				+	+	17.5
					+	20.3
			+		+	23.2
					+	27.5
					+	32
			+	+		32.5
	+	+		+		32.7
			+	+		33.2
		+		+		33.6
			+			35.3
			+	+		37.3
					+	41.4-48.5
				+	+	51.6
		+	+	+	+	54.4
			+			57.6
			+		+	59.8
		+			+	61.5
		+			+	65.7
					+	69.5-80.6
	+	+	+			82.3
				+	+	85.1
					+	87.9
					+	89
				+	+	93.4
					+	107.8
Gentamicin	1 µg/ml			4 µg/ml		
Tetracycline	1 µg/ml				16 µg/ml	

a Inducing agent: AA = acetic acid, CA = citric acid, LA = lactic acid, TE = tetracycline.
b Final induction concentrations: acetic acid 40 mM, citric acid 20 mM, lactic acid 40 mM, tetracycline 16 µg/ml.