



UNIVERSITY of the
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**Sample preparation methods and molecular based
detection for the rapid isolation and identification of
Listeria monocytogenes in food samples**



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A thesis submitted in partial fulfillment of the requirements for the degree of
Magister Scientiae

In the Department of Biotechnology
University of the Western Cape

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2006

DECLARATION

I, the undersigned, declare that ‘**Sample preparation methods and molecular based detection for the rapid isolation and identification of *Listeria monocytogenes* in food samples**’ is my own work and that all sources I have used or quoted have been indicated and acknowledged by means of complete references.

Miss Diane Rip

Signature.....

Date.....

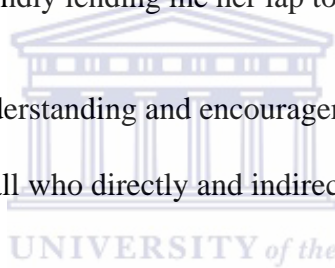


This thesis represents a compilation of articles where each chapter is an individual entity and some repetition between chapters has been unavoidable.

The style of this thesis is in accordance with that of the *Journal of Applied Microbiology*

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Sample preparation methods and molecular based detection for the rapid isolation and identification of *Listeria* *monocytogenes* in food products

CONTENTS

Acknowledgements.....	i
Content page.....	ii
List of figures.....	v
List of tables.....	ix
List of abbreviations.....	x
List of conference contributions emanating from the work reported herein.....	xiii
 CHAPTER 1 Introduction.....	 1
 CHAPTER 2 Literature Review.....	 4
2.1 Introduction.....	4
2.2 Food-borne listeriosis.....	10
2.3 Growth of <i>Listeria</i> in foods.....	11
2.3.1 Contamination of dairy products.....	12
2.3.2 Contamination of meat and poultry.....	15
2.3.3 Contamination of seafood.....	17
2.3.4 Contamination of vegetables.....	20
2.4 Classification.....	21
2.4.1 Serotyping.....	21
2.5 Pathogenicity.....	23
2.5.1 Virulence factors.....	24
2.5.1.1 Listeriolysin O and phospholipases.....	24
2.5.1.2 Actin Polymerizing Protein.....	26
2.5.1.3 Protein p60.....	27
2.5.1.4 PrfA Regulon.....	27

2.5.2	Entry into host cells.....	29
2.5.2.1	Invasion of intestine.....	29
2.5.2.2	Invasion of liver.....	30
2.5.2.3	Invasion of placenta.....	31
2.5.2.4	Invasion of brain.....	31
2.6	Detection methods for <i>Listeria</i>	33
2.6.1	Conventional (traditional) methods.....	33
2.6.1.1	Diluting a sample.....	33
2.6.1.2	Pre-enrichment.....	34
2.6.1.3	Selective enrichment broth.....	36
2.6.1.4	Selective plating.....	36
2.6.1.5	Gram stain and API- <i>Listeria</i>	38
2.6.2	Molecular based detection.....	41
2.6.2.1	Detection of micro-organisms by the PCR reaction.....	41
2.6.2.2	PCR facilitators.....	42
2.6.2.3	PCR inhibitors.....	43
2.6.2.4	Internal amplification control	43
2.6.2.5	Denaturing gradient gel electrophoresis.....	44
2.7	Microbial food-borne hazards.....	47
2.8	HACCP as a good hygienic measure.....	47
2.8.1	The seven principles of HACCP.....	49
2.9	Conclusion.....	49

CHAPTER 3 Evaluation of the factors affecting the sensitivity of *L. monocytogenes* isolation and PCR detection in food products

3.1	Abstract.....	51
3.2	Introduction.....	53
3.3	Materials and Methods.....	56
3.4	Results and Discussion.....	59
3.5	Conclusion.....	70

CHAPTER 4 Internal amplification control design using multiplex PCR for the detection of *Listeria monocytogenes* in food products

4.1	Abstract.....	72
4.2	Introduction.....	74
4.3	Materials and Methods.....	78
4.4	Results and Discussion.....	87
4.5	Conclusion.....	98

CHAPTER 5 Differentiation of the *Listeria* genus in food products by the denaturing gradient gel electrophoresis method

5.1	Abstract.....	100
5.2	Introduction.....	102
5.3	Materials and Methods.....	106
5.4	Results and Discussion.....	114
5.5	Conclusion.....	125



CHAPTER 6 Conclusion.....127

CHAPTER 7 References.....132

Annexure 1	<i>Listeria monocytogenes</i> genome.....	147
Annexure 2	pUC19 genome.....	148
Annexure 3	The results of the nucleotide-nucleotide BLAST indicating the alignment of the 5' end of the iacF primer to <i>L. monocytogenes</i> and the 3' end of the iacF to various pUC19 cloning vectors.....	149
Annexure 4	The results of the nucleotide-nucleotide BLAST indicating the alignment of the 5' end of the iacR to <i>L. monocytogenes</i>	154

LIST OF FIGURES

Figure 2.1	Implicated routes of transmission for <i>L. monocytogenes</i> infection in humans	11
Figure 2.2	Growth and survival of <i>L. monocytogenes</i> in certain foods at 4°C and -20°C.....	17
Figure 2.3	An electron micrograph showing how <i>L. monocytogenes</i> enters and lyses the host cell with the aid of virulence factors	26
Figure 2.4a	<i>L. monocytogenes</i> infection cycle.....	30
Figure 2.4b	The pathogenicity of <i>L. monocytogenes</i>	30
Figure 2.5	An electron micrograph showing a 35 minute post-infection of HBMEC with <i>L. monocytogenes</i>	32
Figure 2.6	Generic method for the isolation and detection of <i>L. monocytogenes</i> in food products.....	40
Figure 2.7	The detection of micro-organisms by PCR is usually divided into 4 stages, namely sample collection, sample preparation, DNA amplification and detection.....	42
Figure 2.8	Illustration of the movement of DNA in denaturing gradient gels.....	46

Figure 3.1	Comparison of two different enrichment broths for the detection of <i>L. monocytogenes</i> from spiked ostrich meat samples.....	60
Figure 3.2	Evaluation of Bioline <i>biotaq</i> , Promega <i>Taq</i> and Roche's <i>Tth</i> to determine the one most efficient in overcoming the effect of inhibitors.....	62
Figure 3.3	The minimum number of <i>L. monocytogenes</i> ScottA in ostrich meat detectable by the PCR reaction.....	64
Figure 3.4	The minimum number of <i>L. monocytogenes</i> NCTC 4855 in ostrich meat detectable by the PCR reaction.....	64
Figure 3.5	The assessment of the minimum number of <i>Listeria monocytogenes</i> ScottA in cheese, hake and minced meat that was detected by the PCR reaction.....	65
Figure 3.6	Determination of the detection limit of <i>L. monocytogenes</i> ScottA within spiked ostrich meat samples.....	66
Figure 3.7	Determination of the detection limit of <i>L. monocytogenes</i> ScottA within spiked camembert cheese samples.....	67
Figure 3.8	<i>L. monocytogenes</i> isolated and detected from a naturally contaminated camembert cheese sample.....	69
Figure 4.1	Schematic representation for the construction of the IAC.....	82
Figure 4.2	The PCR result obtained when using one primer set (hlyF and hlyR).....	88

Figure 4.3	The PCR result obtained when using one primer set (hlyF and hlyR) and touchdown gradient PCR.....	89
Figure 4.4	The results obtained from multiplex PCR with primer sets iacF, iacR, LmonoF and LmonoR in order to determine the optimal concentration of pUC19 for its co-amplification with <i>L. monocytogenes</i>	90
Figure 4.5	Co-amplification of a pure culture of <i>L. monocytogenes</i> ScottA with an optimal concentration of pUC19 DNA using multiplex PCR.....	92
Figure 4.6	A camembert cheese sample spiked with <i>L. monocytogenes</i> ScottA and co-amplified with pUC19 at a concentration of 0.001 pg μl^{-1}	93
Figure 4.7	Assessment of the detection limit of <i>L. monocytogenes</i> in the absence of the pUC19 IAC.....	94
Figure 4.8	The assessment of the inhibitory effect of pUC19 at a concentration of 0.001 pg μl^{-1} on the detection limit of <i>L. monocytogenes</i>	95
Figure 4.9	Evaluation of the inhibitory effect of a higher concentration of pUC19 on the detection limit of <i>L. monocytogenes</i> isolated from spiked ostrich meat.....	96
Figure 5.1	Agarose gel electrophoresis of the products obtained after amplification of the <i>iap</i> gene from <i>L. monocytogenes</i> serotypes and <i>L. innocua</i>	118
Figure 5.2	DGGE profile of <i>Listeria</i> pure cultures and spiked food products	119

- Figure 5.3** Gradient PCR of an ostrich meat sample spiked with *L. monocytogenes* NCTC 7973 using the *iap* gene as a target for PCR amplification.....120
- Figure 5.4** Result of the optimized PCR protocol and PCR cycle parameters for the amplification of pure cultures of *Listeria*.....121
- Figure 5.5** DGGE analysis of *Listeria* pure cultures and spiked food samples on a polyacrylamide gel using the PCR products from the optimized PCR protocol and cycle parameters.....122



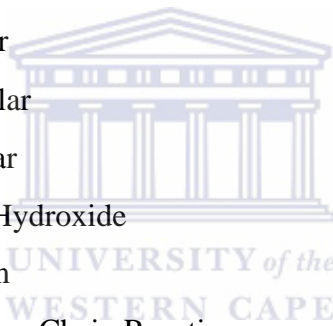
LIST OF TABLES

Table 2.1	Specifications for the presence of <i>L. monocytogenes</i> in various food products as indicated by the Department of Health, South Africa.....	9
Table 2.2	Conditions for pathogen growth.....	12
Table 2.3	Time/Temperature guidance for controlling pathogen growth and toxin formation in seafood.....	20
Table 2.4	Serotypes of <i>Listeria</i> species.....	23
Table 2.5	Virulence genes regulated by the PrfA Regulon.....	28
Table 3.1	Sequence of the primer set specific for the amplification of the <i>hly</i> gene of <i>L. monocytogenes</i>	58
Table 4.1	Construction of an internal amplification control (IAC) for the simultaneous amplification of <i>L. monocytogenes</i> and pUC19.....	81
Table 4.2	Melting temperature (T _m) and GC content of primers designed for IAC.....	81
Table 5.1	Primer set used for PCR-DGGE analysis.....	112
Table 5.2	A comparison of the reference strains subjected to biochemical and molecular methods.....	116

LIST OF ABBREVIATIONS

- Act A Actin Polymerizing protein A
- API Analytical profile index
- a_w Water activity
- AT Adenine-Thymine
- β Beta
- bp base pair
- BPW buffered peptone water
- BSA Bovine serum albumin
- C Cytosine
- CCP Critical Control Point
- CFSAN Center for Food Safety and Applied Nutrition
- cfu g⁻¹ Colony forming units per gram
- cfu ml⁻¹ Colony forming units per milliliter
- dATP Deoxyadeninetriphosphate
- dCTP Deoxycytosinetriphosphate
- dGTP Deoxyguaninetriphosphate
- DGGE Denaturing Gradient Gel Electrophoresis
- dNTP Deoxynucleotidetriphosphate
- dTTP Deoxythyminetriphosphate
- *et al.* and others
- FB Fraser broth
- FDA Food and Drug Administration
- FSIS Food Safety and Inspection Service
- G Guanine
- GC Guanine-Cytosine
- h hour

- HACCP Hazard Analysis and Critical Control Points
- Hpt Hexose phosphate transporter
- IAC internal amplification control
- Inl Internalin
- kDa kilo Dalton
- LEB Listeria enrichment broth
- LLO listeriolysin O
- min minutes
- min. minimum
- max. maximum
- µl microliter
- µM micromolar
- mM millimolar
- NaOH Sodium Hydroxide
- ng nanogram
- PCR Polymerase Chain Reaction
- pg picogram
- PLC's Phospholipase C
- PrfA Positive regulatory factor A
- rDNA ribosomal DNA
- rpm revolutions per minute
- RTE Ready To Eat
- SDS Sodium Dodecyl Sulphate
- s seconds
- spp. species
- *Taq* *Thermus aquaticus*
- temp temperatures
- *Tth* *Thermus thermophilus*



- USDA United States Department of Agriculture
- US/DHHS United States Department of Health and Human Services
- USFDA United States Food and Drug Administration
- v/v volume per volume
- < less than
- 1° primary
- 2° secondary



LIST OF CONFERENCE OUTPUTS EMANATING FROM THE WORK REPORTED HEREIN

Gouws, P. A. and **Rip, D.** (2005) PCR based detection of *Listeria monocytogenes* in complex food samples. Bio Rad Food Safety Symposium: Cape Town, South Africa

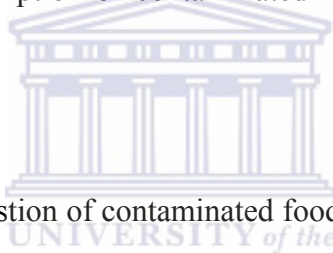
Gouws, P. A. and **Rip, D.** (2006) The effect of different *Taq* and *Tth* polymerases in the detection of *Listeria monocytogenes* in food. ICFMH, FoodMicro: Bologna, Italy



CHAPTER 1

1.1 INTRODUCTION

Listeria monocytogenes is a Gram-positive bacterium responsible for listeriosis, a food-borne disease, which may result in severe illness and possible death. The importance of *L. monocytogenes* as a food-borne pathogen has been recognized since the 1980's when a correlation between the consumption of contaminated foodstuffs and human listeriosis outbreaks was observed.



Listeriosis occurs with the ingestion of contaminated foods. The death toll is known to be the highest of all known food-borne pathogens, although the disease (listeriosis) is rare. Contamination of food-products with *L. monocytogenes* occurs sporadically in South Africa. To monitor the incidence of *L. monocytogenes* in foods, reliable methods must be developed in order for the organism to be detected rapidly, since there are zero-tolerance specifications for the presence of *L. monocytogenes* in certain food products.

Conventional enrichment and detection methods for *Listeria* in food products are generally reliable yet expensive, time consuming and provide presumptive identification. Molecular approaches for DNA isolation and identification have shown to be faster and more reliable. Methods such as the polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) have been employed to overcome limitations caused

by conventional techniques. There are still, however, problems with the sensitivity and specificity of the PCR reaction. The removal of inhibitory substances is a major step in the preparation of samples for PCR-based detection of food pathogens. The detection of *L. monocytogenes* in food products results in economic consequences for the manufacturer as a result of having to re-call and withdraw contaminated products. A detection method with better performance that reduces time and cost requirements would thereby be of great value to the food industry.

The aim of this study involved developing DNA based methods to aid the food industry for the fast detection of *L. monocytogenes* in food products. Therefore assays were developed in such a way that they will have potential applications in the food industry. The detection of bacteria in pure cultures using the PCR reaction is specific and rapid, but in complex food samples inhibition of the PCR reaction is likely to occur. Pre-PCR treatments were developed and compared to concentrate DNA or target cells and to minimize or reduce inhibition that may block or reduce DNA amplification. The low levels of the pathogen as well as the inhibitors in the food product may lead to a failed PCR reaction resulting in a false negative result.

After optimization of the pre-PCR and PCR reaction, the efficacy of different DNA polymerases were compared in their ability to amplify the gene of interest and overcome the effect of inhibitors. *Taq* DNA polymerase and *Tth* DNA polymerase were evaluated for their ability to overcome inhibition in food products.

A problem that may arise with the PCR reaction is that false-negative results may arise. An internal amplification control (IAC) was designed to detect failure of the amplification reaction. The IAC is a non-target DNA sequence that was amplified together with the target sequence under the same conditions. To eliminate false-negative results, the amplification of the IAC was monitored. The amplification of the IAC indicated that the PCR reaction was not inhibited. The IAC was incorporated into the PCR reaction without loss of specificity and sensitivity.

The subdivision of the genus *Listeria* into serotypes has proven useful for practical and epidemiological purposes, since members of the genus have been implicated in listeriosis outbreaks. Biochemical standard methods for species identification has its limitations since differentiation between species is not always achieved together with the fact that the process is time consuming and laborious. The DGGE technique was employed to differentiate between different species of the genus *Listeria* within food products.

The primers that were used in this study were designed for the amplification of a 730bp region of the *hly* gene. This gene codes for the hemolysin listeriolysin O and is specific to *L. monocytogenes*. *Hly* gene contributes to the pathogenic character of *L. monocytogenes* since it assists with its invasion and replication in host cells. For DGGE, the primers that were used amplify the *iap* gene encoding the invasion-associated protein p60 common in all *Listeria* species.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

The genus *Listeria* comprises 6 characterized species, namely *Listeria monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. grayi* and *L. welshimeri*. This sub-division has proven useful for practical and epidemiological purposes especially problems associated with food-borne listeriosis (Vázquez-Boland *et al.* 2001b; Cocolin *et al.* 2002; Schmid *et al.* 2005; Hain *et al.* 2006). Of these species, *L. monocytogenes* is the only human and animal pathogen capable of causing severe infections like septicemia, meningitis, perinatal infections, encephalitis and gastroenteritis and has been associated with abortions (Cox *et al.* 1998; Bubert *et al.* 1999; Jeffers *et al.* 2001; Dussurget *et al.* 2004; Ward *et al.* 2004). Other than *L. monocytogenes*, *L. ivanovii* the only species of the genus considered important as a pathogen infecting animals, particularly sheep (ruminants) (Axelsson and Sorin 1998; Vázquez-Boland *et al.* 2001a; Domínguez-Bernal *et al.* 2006) and causing listeriosis, which is mainly transmitted by the consumption of spoiled silage (Hain *et al.* 2006).

L. monocytogenes is a non spore-forming, Gram-positive, chemoorganotrophic, facultative anaerobic rod that causes severe human food-borne disease (Farber and

Peterkin 1991; De Cesare *et al.* 2001; Jiang *et al.* 2006) of which incidences in reported cases have increased in the last few decades (Holko *et al.* 2002; Choi and Hong 2003). Members of the *Listeria* genus are small (0.4-0.5 μm in diameter and 0.5-2.0 μm in length) with peritrichous flagella that make the organism motile. They have psychrophilic characteristics and outgrow most other bacteria at 4°C (Schlech 2000) although they have optimal growth at 30°-37°C (Pearson and Marth 1990). They are catalase positive and oxidase negative and display β hemolysin activity, which produce clear zones on blood agar (Pearson and Marth 1990; Farber and Peterkin 1991; Axelsson and Sorin 1998). The ability of *L. monocytogenes* to grow at refrigeration temperature results in its presence being a lethal threat to the food industry e.g. the dairy industry (Cox *et al.* 1998), where soft cheeses and unpasteurized milk are susceptible. The growth of *Listeria*, more importantly, *L. monocytogenes* in a cold environment (refrigeration temperatures) also makes deli meats and cold cuts possible vehicles for food-borne listeriosis and a significant threat to the safety of ready-to-eat (RTE) meat products (Zhu *et al.* 2005).

Listerial infections are dangerous particularly to immuno-compromised individuals, pregnant women, the elderly and newborns (Cox *et al.* 1998). Healthy children and adults occasionally get infected with *Listeria*, but rarely become seriously ill (CDC 2005).

L. monocytogenes is a ubiquitous organism, isolated from a variety of sources, namely soil, plant and vegetation sources, water samples and human and animal feces (Bubert *et al.* 1999; Cocolin *et al.* 2002; Nightingale *et al.* 2005; Valero *et al.* 2006). The natural

habitat of *Listeria* is considered to be the surface layer of soil, which is rich in decaying plant matter (Vázquez-Boland *et al.* 2001a). Large outbreaks of listeriosis have been associated with contaminated foodstuffs including raw vegetables, milk, meat products, various cheeses and seafood, ice cream and chocolate (Boerlin *et al.* 1997; Doyle 2001; Bremer *et al.* 2003; Choi and Hong 2003; Lafarge *et al.* 2004; Rijpens and Herman 2004). Over the last few years, listeriosis outbreaks have been the leading cause of food recalls due to microbiological concerns (Ward *et al.* 2004).

The ubiquitous nature and the ability of *L. monocytogenes* to grow at refrigeration temperatures is a challenge to the food industry. Some countries have set legal limits pertaining to the number of organisms, or *L. monocytogenes* that is allowed in food products, especially RTE food products. However some countries, where no legal limits have been set, have provided guidelines or criteria for the number of *L. monocytogenes* allowed in food products (Nogva *et al.* 2000). The United States Food and Drug Association (USFDA) have issued a zero-tolerance ruling for the presence of *L. monocytogenes* 25 g⁻¹ in RTE foods namely, products that may be eaten without further cooking or heating (Norton *et al.* 2001; FDA/CFSAN *et al.* 2003). Since high levels of *Listeria* are difficult to eradicate in food processing environments, the International Commission on Microbiological Specifications for food specified that 100 cfu g⁻¹ of *L. monocytogenes* in food was allowed at the time of consumption for non-risk consumers (Rodríguez-Lázaro *et al.* 2004b). When a food product is found to be contaminated, the implicated food product is re-called. The United States Department of Agriculture and

Food Safety and Inspection Service (USDA/FSIS) has also issued a zero-tolerance ruling for the presence of *L. monocytogenes* 25 g⁻¹ in RTE food products whereas some countries, including partners in the United States such as Canada and Denmark, have a non zero-tolerance for *L. monocytogenes* pertaining to certain foods. In Canada, RTE foods that have not been linked to an outbreak and show no *L. monocytogenes* growth within a 10-day period of refrigeration storage may contain up to 100 cfu g⁻¹ but cannot exceed 100 cfu g⁻¹. The Canadian policy for *L. monocytogenes* in RTE foods is based on the principles of HACCP (FDA/CFSAN *et al.* 2003; Zhu *et al.* 2005).

With regard to Australia's National Food Standard Council, regulations require that pâté, soft cheeses, smoked fish and smoked sea-food have zero *Listeria* at the point of wholesale distribution. Also, the control of *Listeria* in the dairy industry sets out procedures to prevent *Listeria* contamination in the processing plant namely, milk and milk products and thereby clear a product intended for sale should *Listeria* be found in the dairy product or the environment of the processing plant (Victorian Government Health Information 2004). The Food Standards Australia New Zealand (FSANZ) issued a zero-tolerance ruling for cooked crustacea and processed molluscs and allows < 100 cfu g⁻¹ in one out of five samples of RTE processed finfish (Bremer *et al.* 2003).

Extensive work has been done in Europe to reduce the incidence of listeriosis (Lundén *et al.* 2004). According to the European Community Directive for milk and milk-based products, a zero-tolerance ruling for the presence of *L. monocytogenes* in soft cheeses has

been issued and the organism must be absent in 1 g of other products (Nogva *et al.* 2000). The European Commission has issued a ruling for a maximum level of 100 cfu g⁻¹ in RTE foods at the end of shelf life (Valero *et al.* 2006).

With regard to South African policy, the Department of Health's legislation specified the following; some foods permit the growth and proliferation of *Listeria* with regard to certain factors e.g. storage temperature. For these foods, the limit for the presence of *L. monocytogenes* should be zero. For foods that do not support the growth of *Listeria* the limit is 100 cfu g⁻¹. The Department of Health specifies the following with regard to various food items (Table 2.1) (Department of Health 2001).



Table 2.1 Specifications for the presence of *L. monocytogenes* in various food products as indicated by the Department of Health, South Africa (Department of Health, 2001).

<i>L. monocytogenes</i>	Food item	Limits
	Cheese	0 g ⁻¹
	<u>Cold meal items:</u> Cold meats, processed meats, polony, dried vegetables, ham, potato salad with mayonnaise.	< 10 g ⁻¹
	<u>Cold smoked or fermented meal items:</u> Salami, bacon, buns, bread, smoked cold meat, caviar.	
	<u>Items requiring further cooking:</u> Blanched and frozen vegetables, half-cooked meals (also steak, chops, wors), raw meat, meat basting sauce.	< 1000 g ⁻¹ < 100 g ⁻¹

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The United States Department of Health and Human Services (US/DHHS) co-ordinated a ‘Healthy people 2010’ project worked on by the FDA, FSIS and Centre for Disease Control (CDC) in order to achieve an additional 50% reduction in listeriosis by the year 2010. This initiative aims to prevent disease and promote health by bringing together various organizations, government and communities to improve the quality of life (FDA/CFSAN *et al.* 2003). The reduction/absence of *L. monocytogenes* in food products will prevent economic consequences like the withdrawal of products leading to a decrease in sales for products suspected of being contaminated. Usually the presence of any *Listeria* species in food is an indicator of poor hygiene (Cocolin *et al.* 2002).

2.2 FOOD-BORNE LISTERIOSIS

Listeriosis is acquired by eating foods that is contaminated with *Listeria* (Zhou and Jiao 2005). Although the incidence of infection is low, listeriosis has a high mortality rate especially within vulnerable groups namely the elderly, infants and the immuno-compromised (Elliot and Kvenberg 2000; Lundén *et al.* 2004; Besse *et al.* 2005; Nappie *et al.* 2005). The occurrence of listeriosis is quite low with 2-15 cases per million in the USA, although the mortality rate is about 20-30% and a 90% hospitalization rate for those with listeriosis (Kwiatek 2004; Zhang *et al.* 2004; Hain *et al.* 2006) compared to a 0.04% death rate with food-borne salmonellosis (Doyle 2001). The minimal infective dose is estimated to be $> 100 \text{ cfu g}^{-1}$ with listeriosis cases being sporadic but occasionally also epidemic (Department of Health 2001; Holko *et al.* 2002).

Listeriosis has always been regarded as an invasive disease affecting susceptible groups, but a non-invasive form of listeriosis that causes febrile gastroenteritis, headaches, nausea and vomiting in healthy adults has increased public awareness of *L. monocytogenes* due to the expanding vehicle of infection (Franciosa *et al.* 2001; Lundén *et al.* 2004; Nappi *et al.* 2005).

2.3 GROWTH OF *LISTERIA* IN FOODS

Listeria monocytogenes and *L. ivanovii*, species of the genus *Listeria* are both commonly found in rotting vegetation, soil and water. Only the species *L. monocytogenes* been recognized as a human pathogen responsible for several epidemics of listeriosis. Several sources have been identified as possible routes for *L. monocytogenes* transmission to humans (Figure 2.1) (Axelsson and Sorin 1998). Since *Listeria* can survive and grow under adverse conditions including a low pH, low refrigeration temperature and high salt concentration (Table 2.2), they easily contaminate food and become a concern to the food industry (Lundén *et al.* 2004; Burnett *et al.* 2005). The fact that *L. monocytogenes* has been implicated in outbreaks and sporadic cases due to their prevalence in dairy and meat products has resulted in serious economic losses due to product recalls (Besse *et al.* 2004; Leite *et al.* 2006).

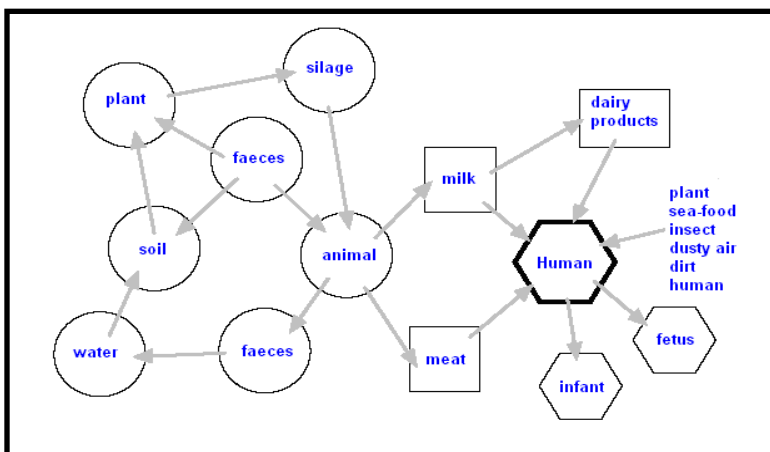


Figure 2.1 Implicated routes of transmission for *L. monocytogenes* infection to humans (adapted from Axelsson and Sorin 1998)

Table 2.2 Conditions for pathogen growth (adapted from Bremer *et al.* 2003)

Pathogen	Conditions for Pathogen Growth						Oxygen requirements
	min. a_w	min. pH	max. pH	max. % salt	min. temp.	max. temp.	
<i>Listeria monocytogenes</i>	0.92	4.8	9.6	10	0-2°C	45°C	facultative anaerobe

2.3.1 Contamination of dairy products

Listeriosis outbreaks have been associated with dairy products manufactured from raw milk (Lundén *et al.* 2004). The ability of *Listeria monocytogenes* to contaminate dairy food is a lethal threat to the dairy industry. Dairy products such as raw milk samples and soft cheese have shown to be vehicles of contamination during *L. monocytogenes* outbreaks and the number of outbreaks associated with dairy products accounts for half the number of outbreaks caused by all food types (Cox *et al.* 1998).

Milk and milk products are highly perishable foods that may be potentially unsafe to consume due to the growth of micro-organisms (Xanthiakos *et al.* 2006). Raw/unpasteurized milk or food made from raw milk may contain *L. monocytogenes* (Schett *et al.* 2005). *Listeria* are killed by pasteurization, however contamination may occur after pasteurization but before packaging (CDC 2005). An example is butter made from pasteurized milk, which proved to be contaminated in subsequent stages of

production (Lundén *et al.* 2004). An increase in the number of listeriosis cases in Northern England was linked to the consumption of contaminated butter (Greenwood *et al.* 2005).

Soft cheeses manufactured from pasteurized milk have also been linked to listeriosis outbreaks suggesting that raw milk is not the only risk product (Lundén *et al.* 2004). Although the pasteurization of raw milk is considered as being efficient to control/prevent *L. monocytogenes* contamination, chances are that the product may become contaminated after this heat treatment. An outbreak in 1994 in Illinois caused 45 people to become ill. The outbreak was linked to chocolate milk that contained *L. monocytogenes* serotype 1/2 b (Doyle 2001). Factors that influence milk composition and microbial load include hygienic practices of farmers namely, the washing of milking equipment and udder preparation for milking. Intensive washing of milking equipment and udder preparation may result in raw milk containing spoilage and/or pathogenic micro-organisms. Where the milk is stored at a low temperature, *Pseudomonas* spp. together with *Listeria* may proliferate. Where the udder is not cleaned efficiently, salt tolerant micro-organisms tend to proliferate (Lafarge *et al.* 2004). Where milk and milk products are not properly processed, packaged, distributed and stored, micro-organisms may proliferate and make the product unsafe for consumption (Xanthiakos *et al.* 2006).

One of the food products most frequently associated with listeriosis are soft cheeses, especially those made from unpasteurised milk (Holko *et al.* 2002). A heat treatment that is given during cheese manufacturing is usually sufficient in killing *L. monocytogenes*

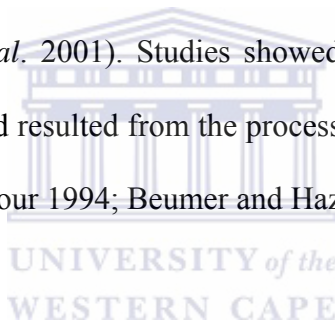
that may be present, although post-processing contamination is a possibility (Longhi *et al.* 2003). *L. monocytogenes* tolerates harsh conditions and therefore can grow and survive in different types of food. *L. monocytogenes* tolerates conditions such as a low a_w and a high salt concentration, which facilitates its survival in products with a high fat content, example semi-hard cheese (Lundén *et al.* 2004). The growth and survival of *L. monocytogenes* in soft cheeses is favoured by their maturation and storage at refrigeration temperatures (Leite *et al.* 2006).

The prevalence of *L. monocytogenes* in different types of cheeses varies. In soft and semi-soft cheeses the a_w is higher than in hard cheeses, facilitating faster growth for the organism whereas in mold cheese, during the ripening process, pH levels approach neutral facilitating the growth of *L. monocytogenes* (Lundén *et al.* 2004). During ripening, an increase in the pH of cheese is paralleled by an increase in the growth of the organism, which results in contamination being centered on the surface of the rind (Pearson and Marth 1990; Farber and Peterkin 1991). *L. monocytogenes* can survive a number of cheese-making processes (manufacturing and ripening) if present in raw milk (100 cfu ml^{-1}) and remain viable in the final product for a long time. It survives best in cheeses such as camembert and worst in cottage cheese. *L. monocytogenes* tends to be concentrated in the curd and scarcer in the whey (Griffiths 1989; Pearson and Marth 1990; Farber and Peterkin 1991).

Healthy cows can serve as reservoirs for *L. monocytogenes* whereby the organism is secreted in its milk. Milk can also become contaminated through accidental contact with feces and silage (Axelsson and Sorin 1998).

2.3.2 Contamination of meat and poultry

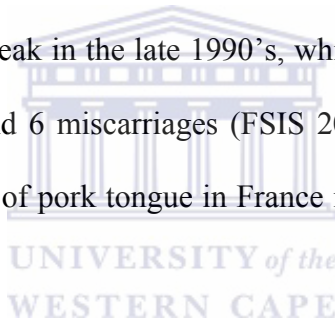
Listeria monocytogenes has been associated with a variety of foods including RTE foods and is a well-known problem in production environments including abattoirs and meat processing plants (Purwati *et al.* 2001). Studies showed that *L. monocytogenes* strains isolated from meat or RTE food resulted from the processing environment rather than the animal itself (Harvey and Gilmour 1994; Beumer and Hazeleger 2003).



Meat and meat products have frequently been contaminated with *L. monocytogenes* where the organism has shown to proliferate through frozen foods (Mahmood *et al.* 2003) and poses a risk to the safety of RTE meat products (Figure 2.2) (Purwati *et al.* 2001; Cocolin *et al.* 2004; Rodríguez-Lázaro *et al.* 2004b; Zhu *et al.* 2005). *L. monocytogenes* began to emerge as a problem associated with processed meat and poultry during the 1980's (FSIS 2003) and since has been commonly isolated from different types of processed meats where it has shown to have an increase in growth in high pH cooked meat and poultry products (Samelis and Metaxopoulos 1999). It has been shown that *L. monocytogenes* grows fairly well in meats and poultry products with a pH near or above 6.0 and poorly or not at all below pH 5.0 (Zhu *et al.* 2005). Low pH cured meats e.g.

fermented sausages do not pose much risk to the consumer since only a few *L. monocytogenes* cells may survive in the product. The use of poultry meat to produce processed meats has paralleled an increase in the level of *Listeria* in meat products as chicken, turkey breasts, wings and drumsticks have shown a high incidence of haemolytic and non-haemolytic *Listeria* spp. (Samelis and Metaxopoulos 1999).

Frankfurters has been a source of *L. monocytogenes* food poisoning when a cancer patient died developing meningitis (Mahmood *et al.* 2003). Luncheon meats and hotdogs were implicated in a listeriosis outbreak in the late 1990's, which resulted in 101 illnesses and 21 deaths - 15 adult deaths and 6 miscarriages (FSIS 2003). An outbreak of listeriosis was linked to the consumption of pork tongue in France in 1999/2000 (Greenwood *et al.* 2005).



RTE cooked meats are commonly contaminated with *L. monocytogenes* during slicing and packaging after cooking or during the post-processing steps which is a concern since RTE cooked meats that do not require further cooking have a long shelf life and *L. monocytogenes* also has the ability to grow at refrigerated temperatures in the presence of a high salt concentration used for preserving meat products (Mahmood *et al.* 2003; Kwiatek 2004; Bruhn *et al.* 2005; Zhu *et al.* 2005). In a study undertaken by Samelis and Metaxopoulos (1999), *Listeria* were not detected in sausages heated in their final packs neither in the fully ripened dry salamis suggesting that contamination was likely to occur after cooking or during post-processing steps. Fresh sausages are very perishable

products with a a_w equal to or higher than 0.97 and a pH value not lower than 5.5 (Cocolin *et al.* 2004).

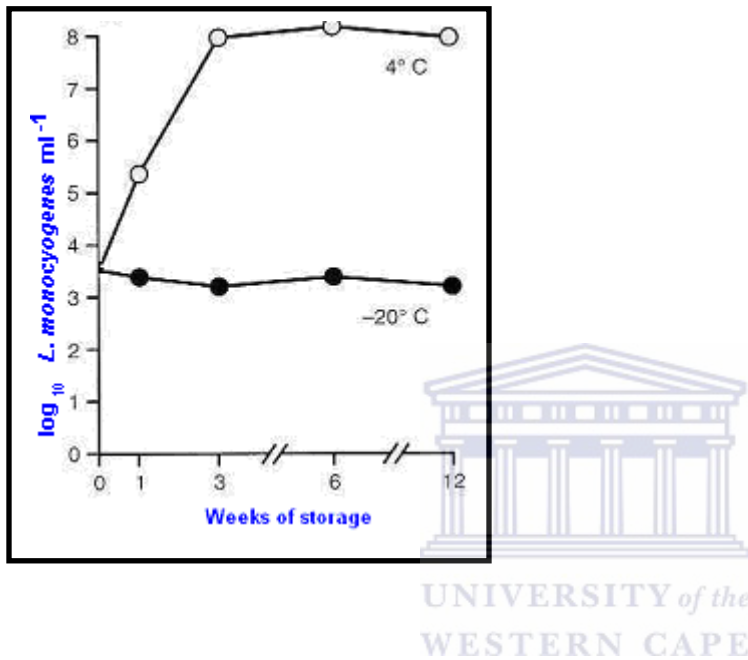


Figure 2.2 Growth and survival of *L. monocytogenes* in certain foods at 4°C and -20°C. (Adapted from Todar 2003)

2.3.3 Contamination of seafood

The consumption of seafood contaminated with *Listeria monocytogenes* has been implicated with human listeriosis (Agersborg *et al.* 1997; Kwiatek 2004). *L. monocytogenes* has been isolated from fresh, frozen and processed products including crustaceans, molluscan shellfish and finfish (Elliot and Kvenberg 2000; Bremer *et al.* 2003). A time / temperature guidance for controlling pathogen growth and toxin formation is seen in Table 2.3.

Cold smoked fish products and hot smoked mussels are RTE food products usually eaten without cooking, and have posed a risk for contamination with *L. monocytogenes* since there is a lack of a heat inactivation step during processing (Boerlin *et al.* 1997; Norton *et al.* 2001; Bremer *et al.* 2003; Besse *et al.* 2004). The salt content, pH and a_w levels usually facilitate the growth and survival of *L. monocytogenes* (Rórvik 2000). Cold smoked salmon is a RTE food product that is smoked to add flavour as well as to preserve the product by preventing the growth of micro-organisms. Since this procedure is performed at a temperature below 28°C, it is not always effective in eliminating micro-organisms that may have been on the raw product prior to smoking (Simon *et al.* 1996). The smoking of salmon could involve cold smoking, which tends to reduce the level of *L. monocytogenes* but not eliminate them since the temperature is too low, or hot smoking (where smoke is applied during the whole heating process) at a temperature higher than 60°C to eliminate the micro-organisms (Rórvik 2000).

In a study undertaken by Boerlin *et al.* (1997) to determine whether specific strains of *L. monocytogenes* were specific for particular types of fish products, it was found that there was no association between a specific population of *L. monocytogenes* and sea-food products, however it was found that most of the *L. monocytogenes* isolated from smoked salmon belonged to serotype 1/2 (Rórvik 2000).

A popular sea-food dish in Sweden, 'gravad' rainbow trout, is prepared by mixing rainbow trout with sugar, salt, pepper and dill, where it is placed in a plastic bag and

refrigerated for 2 days. Thereafter, it is vacuum packed and stored at 8°C for up to 21 days. *L. monocytogenes* has been implicated in the contamination of such fish products where it can proliferate at the refrigeration temperature and develop into high numbers (Ericsson and Stålhandske 1997).

L. monocytogenes has shown not only to survive, but increase in numbers when present on vacuum packed smoked sea-food products that is stored at 4° or 10°C. Where shrimp, crabmeat and other sea-food products were artificially contaminated with *L. monocytogenes* and stored at 7°C, their numbers increased by 100 000 bacteria g⁻¹ within 14 days (Bremer *et al.* 2003).

An outbreak of listeriosis in Sweden in the 1990's was linked to the contamination of cold-smoked rainbow trout and 'gravad' trout which caused 2 deaths. A correlation between the ingestion of cold smoked fish and human listeriosis was found since the same *L. monocytogenes* strains were recovered from the fish products and the patients (Besse *et al.* 2004).

Where *L. monocytogenes* contamination has occurred, the cause has commonly been traced to harvesting, handling, processing or sanitation namely, the production environment (Beumer and Haxeleger 2003; Bremer *et al.* 2003). Contaminated processing surfaces have been believed to be the biggest source of *L. monocytogenes* contamination of sea-food. A study undertaken by Autio *et al.* (1999) showed that *L.*

monocytogenes contamination of fish occurred during processing (brining and slicing) contrary to belief that *L. monocytogenes* contamination of cold smoked rainbow trout was a result of the raw fish. Various studies concluded that raw fish is not a major source of contamination; however slaughtered fish from slaughterhouses may introduce the bacteria and contaminate the plant (Rórvik 2000).

Table 2.3 Time/Temperature guidance for controlling pathogen growth and toxin formation in seafood (USFDA/CFSAN 2001)

Time/Temperature Guidance for Controlling Pathogen Growth and Toxin Formation in Seafoods		
Pathogen	Product Temperature	Maximum Exposure Time
Growth of <i>Listeria monocytogenes</i>	-0.4-5°C	7 days
	6-10°C	2 days
	11-21°C	12 h
	above 21°C	3 h

2.3.4 Contamination of vegetables

Listeria monocytogenes occurs on fresh vegetables and can grow and survive on fresh produce stored at refrigeration temperature (Thomas *et al.* 1999; González-Fandos *et al.* 2001). Vegetables can become contaminated from the soil or manure, which is used as fertilizer and has the ability to grow and survive under conditions associated with processing and storage of raw fruit and vegetables (Li *et al.* 2002; CDC 2005). *Listeria* may be present in high numbers on the surface of vegetables where cell tissue has lost its

strength and decay is more prevalent (Beumer and Hazeleger 2003). *L. monocytogenes* has been associated with the consumption of unwashed raw vegetables and cabbage (Mahmood *et al.* 2003). Coleslaw was implicated in a major listeriosis outbreak in Canada and lettuce, celery and tomatoes were implicated in an outbreak in 8 Boston hospitals (Li *et al.* 2002). In New Zealand in 2001, a listeriosis outbreak was linked to unwashed vegetables, salad that had been cross contaminated with raw poultry and some other food products (Bremer *et al.* 2003).

2.4 CLASSIFICATION



2.4.1 Serotyping

Listeria species can be sub-divided into serotypes by means of their antigenic variation namely somatic (O) and flagellar (H) antigens. *Listeria monocytogenes* has 13 serotypes, some of which are common to *L. innocua* and *L. seeligeri* (Table 2.4) (Axelsson and Sorin 1998; Nadon *et al.* 2001; Nightingale *et al.* 2005). Of the 13 serotypes of *L. monocytogenes*, 1/2a, 1/2b and 4b are responsible for 98% of listeriosis infections in humans, hence 98% of isolates belong to only 3 serotypes. The genetic variation among these strains is mainly a result of genes encoding surface proteins, genes involved in sugar metabolism and virulence factors necessary for the infection of the host cell (Farber and Peterkin 1991; De Cesare *et al.* 2001; Cabrita *et al.* 2004; Dussurget *et al.* 2004; Bruhn *et al.* 2005). This subtyping therefore indicates that some strains are more

pathogenic to humans than others (McLauchlin 1987). A study undertaken by Nappi *et al.* (2005) showed that molecular characterization of *L. monocytogenes* by serotyping allowed for the identification of the strains 1/2a, 1/2b and 4b as the most important agents of invasive and non-invasive listeriosis in humans and ruminants.

However, most sporadic human cases and outbreaks have been caused by *L. monocytogenes* serotype 4b, suggesting specific virulence properties in this serotype (De Cesare *et al.* 2001; Cabrita *et al.* 2004; Doumith *et al.* 2004; Dussurget *et al.* 2004). Strains of the antigenic group 1/2 (1/2a, 1/2b, 1/2c) have been reported to predominate in food isolates but have been shown to be increasing in human isolates (Vázquez-Boland *et al.* 2001b; Cabrita *et al.* 2004). The genome of the *L. monocytogenes* serotype 4b isolate (CLIP 80459) has been partially sequenced and compared with the sequences of serotype 1/2a (EGDe) and *L. innocua*. Findings showed that there was a great genetic diversity within the *L. monocytogenes* species where 8% of the 4b serotype genes were absent from the serotype 1/2a genome and the latter, in turn, had 10.5% of its genes absent from the *L. innocua* genome. This emphasized that, although certain *L. monocytogenes* genes were absent from the *L. innocua* genome, it was obviously not required for the virulence of that particular *L. monocytogenes* strain. The work done also showed that genetic variation among *L. monocytogenes* serotypes is similar to that between *Listeria* species (Dussurget *et al.* 2004; Zhang *et al.* 2004; Nightingale *et al.* 2005).

No links have been made between certain forms of listeriosis and specific serotypes, but work has shown that there is an association between perinatal listeriosis and serotypes 1/2a, 3b and 4b (Farber and Peterkin 1991).

Table 2.4 Serotypes of *Listeria* spp. (adapted from Axelsson and Sorin 1998)

SPECIES	SEROTYPE
<i>L. monocytogenes</i>	1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, 7
<i>L. innocua</i>	3, 6a, 6b, 4ab
<i>L. ivanovii</i>	5
<i>L. seeligeri</i>	1/2a, 1/2b, 1/2c, 4b, 4c, 4d, 6b
<i>L. welshimeri</i>	1/2a, 4c, 6a, 6b

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2.5 PATHOGENICITY

Listeria monocytogenes is an intracellular parasite that can invade and replicate in epithelial cells and macrophages. It is capable of crossing three barriers namely the intestinal, blood-brain and placental barriers (Dussurget *et al.* 2004; Lecuit 2005). The fact that *L. monocytogenes* can cause severe illness is due to its ability to induce its own uptake by the host cell and then replicate and spread to other cells. This process is facilitated by the bacterium producing a series of virulence factors for each step of the invasion process (McLauchlin 1997; Greiffenberg *et al.* 2000; Doyle 2001; Vázquez-

Boland *et al.* 2001a). A transcriptional activator PrfA activates all the virulence genes on the cluster and regulates their expression.

2.5.1 Virulence factors

2.5.1.1 *Listeriolysin O and phospholipases*

The ability of *Listeria* to invade and replicate in host cells depends on its virulence genes. The virulence determinants of *Listeria* spp. are clustered along the chromosome in genomic islands. The locus for *L. monocytogenes* consists of 3 transcriptional units. First is the central position - the *hly* monocistron; downstream from *hly* is an operon comprising 3 genes namely, *mpl*, *actA*, *plcB* and upstream from *hly* is the *plcA-prfA* operon (Vázquez-Boland *et al.* 2001a).

The hemolysin gene, *hly*, was the first virulence factor to be recognized and sequenced in *Listeria* spp. and its specific role in the pathogenesis of *Listeria* infection was demonstrated (Farber and Peterkin 1991; Vázquez Boland *et al.* 2001b).

Early evidence that the *Listeria* hemolysin (*hly*) is similar in function and antigenicity to streptolysin O (SLO) from *Streptococcus pyogenes* was provided. Eventually it was confirmed that the hemolysin of *L. monocytogenes* is a SLO-related cytolysin and a

cholesterol-dependent pore-forming toxin (Vázquez-Boland *et al.* 2001b). This toxin was designated listeriolysin O (LLO).

As *L. monocytogenes* invades the host cell, they reside in the vacuole that is surrounded by a membrane. *L. monocytogenes* will almost immediately be killed by phagocytic cells unless they are able to produce LLO and thereby lyse the vacuole and escape into the cytoplasm. LLO is a 60-kDa protein that allows *L. monocytogenes* to escape from the vacuole (Dussurget *et al.* 2004; Jiang *et al.* 2006). Mutants that lack LLO are unable to reach the cytoplasm. The absence of *hly* therefore equals avirulence (Doyle 2001; Vázquez-Boland *et al.* 2001a), thus it can be said that LLO is secreted by all virulent strains of *Listeria*. Once in the cytoplasm, the organism will acquire nutrients from the cytosol and multiply (Figure 2.3). *L. monocytogenes* secretes two Phospholipase C's (PLC's) that are implicated in the lysis of intracellular vacuoles. They act with LLO in facilitating the lyses of primary and secondary vacuoles (Camilli *et al.* 1993). The first PLC is phosphatidylinositol (PI-PLC) specific and the second PLC is phosphatidylcholine (PC-PLC) specific. Studies have showed that PI-PLC assists in the escape of the organism from the primary vacuole whereas PC-PLC is active during cell-to-cell spread of the bacteria (Doyle 2001).

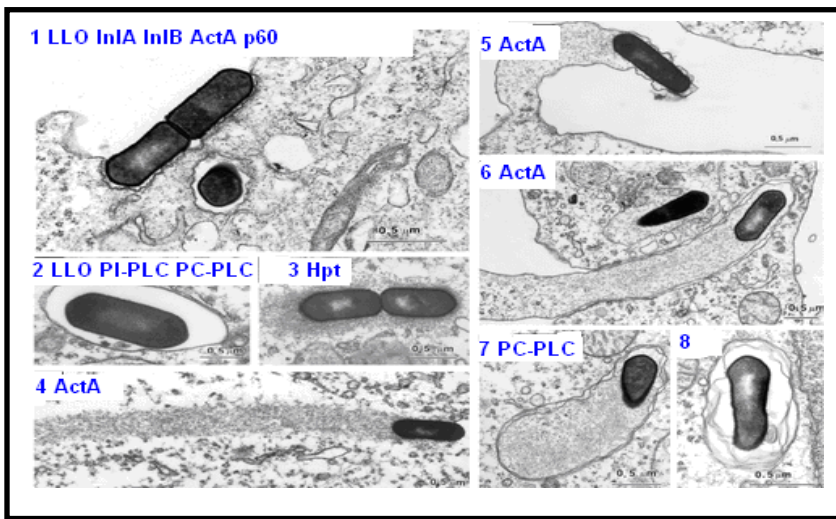


Figure 2.3 An electron micrograph showing how *L. monocytogenes* enters and lyses the host cell with the aid of virulence factors at each step. **1** entry, **2** lysis of the vacuole, **3** intracellular replication, **4** intracellular movements, **5;6** cell-to-cell spread, **7** formation, **8** lysis of the two-membrane vacuole (adapted from Dussurget *et al.* 2004).

2.5.1.2 Actin Polymerizing Protein A (ActA)

Downstream from *hly* is a 5.7kb operon comprising *actA* (1 of 3 genes). The gene *actA* encodes the surface protein ActA (Vázquez-Boland *et al.* 2001a). Once the vacuole has been lysed and *L. monocytogenes* reaches the cytoplasm, they need to reach other cells in order for them to multiply. They do this by means of ActA, a 639 amino acid protein that induces polymerization of globular actin molecules to actin filaments, which allows the bacterium to move from cell to cell along these filaments to the cell membrane. Hence, ActA allows for the mobility of *L. monocytogenes* and attachment and entry into target cells (Kocks *et al.* 1992; Doyle 2001; Jiang *et al.* 2006). ActA is therefore also required for *L. monocytogenes* pathogenicity (Vázquez-Boland *et al.* 2001a).

2.5.1.3 Protein p60 (p60)

The *iap* gene encodes the extracellular protein p60, which is common to all *Listeria* spp. (Kohler *et al.* 1990). It is regarded as an essential murein hydrolase enzyme that facilitates septum separation during the final stage of cell division (Jiang *et al.* 2006). It has been shown that p60 plays a role in the adherence of the bacterium to the host cell (Bubert *et al.* 1999). The *iap* gene was demonstrated to be a reliable PCR target for differentiation of *Listeria* spp. It has conserved regions at the 5' and 3' ends and a species-specific internal region (Cocolin *et al.* 2002).



2.5.1.4 Positive Regulatory factor A (PrfA) Regulon

The most important *Listeria* virulence genes namely, *hly*, *actA*, *prfA*, internalins (*inlA*, *inlB*, *inlC*), hexose phosphate transporter (*hpt*), metalloprotease (*mpl*), *plcA* and *plcB* are regulated by a transcriptional activator PrfA, a protein comprising 233 amino acids. They are known as the PrfA-dependent virulence gene cluster. PrfA is the only regulator identified to date in *Listeria* spp. which is directly involved in the control of virulence gene expression within infected host cells (Table 2.5) (Vázquez-Boland *et al.* 2001a; Dussurget *et al.* 2004). The virulence gene cluster, which is present in *L. monocytogenes*, has been shown to be completely absent from 3 other non-pathogenic serotypes of *Listeria* namely *L. innocua*, *L. welshimeri* and *L. grayi* (Vázquez-Boland *et al.* 2001a).

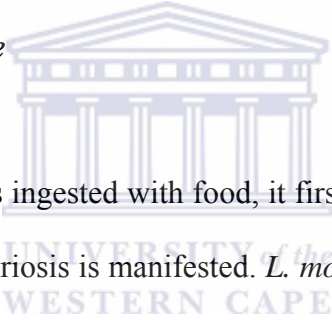
Table 2.5 Virulence genes regulated by the PrfA Regulon

Virulence Genes	Function	Reference
<i>prfA</i>	This gene encodes PrfA, a protein which is necessary to activate all the genes of the cluster and vital to the virulence potential of <i>L. monocytogenes</i> .	Ward <i>et al.</i> 2004; Domínguez-Bernal <i>et al.</i> 2006
<i>inlA</i> and <i>inlB</i>	InlA is a bacterial surface protein encoded by the gene <i>inlA</i> that plays a role in the crossing of the intestinal barrier. InlB is a protein encoded by the gene <i>inlB</i> and acts both as a growth factor and as an invasin. It plays a role in the invasion of hepatocytes in the liver. InlA and InlB are a family of leucine-rich repeat proteins. They were the first factors associated with the invasion of the target cell.	Braun <i>et al.</i> 1998; Greiffenberg <i>et al.</i> 1998; Doyle 2001; Hain <i>et al.</i> 2006
<i>Hpt</i>	<i>Hpt</i> encodes a hexose phosphate transporter (Hpt). It functions as a sugar uptake system that allows bacterial intracellular replication. <i>L. monocytogenes</i> uses phosphate sugar in the cytoplasm to obtain nutrients from the host cell.	Dussurget <i>et al.</i> 2004; Domínguez-Bernal <i>et al.</i> 2006
<i>mpl</i>	The <i>mpl</i> gene encodes an enzyme metalloprotease (Mpl) that processes the immature form of PC-PLC into a mature form. It works with <i>hly</i> , <i>plcB</i> and <i>plcA</i> to disrupt the primary vacuoles after host cell invasion. Mutations in <i>mpl</i> have shown to reduce virulence in a mouse model.	Dreverts 1998; Vázquez- Boland <i>et al.</i> 2001a; Todar 2003
<i>plcA</i>	<i>plcA</i> encodes the protein PlcA (a PI specific PLC). This protein works with <i>hly</i> and <i>plcB</i> to disrupt 1° vacuoles.	Mengaud <i>et al.</i> 1991
<i>plcB</i>	<i>plcB</i> encodes the protein PlcB (a PC specific PLC). Its primary function is to disrupt the double membrane 2° phagosomes formed after cell to cell spread. The 3 protein products from the <i>mpl-actA-plcB</i> operon all assist in cell to cell spread of <i>L. monocytogenes</i> and its escape from the host's immune response in the extracellular compartment.	Dreverts 1998; Vázquez- Boland <i>et al.</i> 2001a-

2.5.2 Entry into host cells

The susceptibility of the host plays a major role in the manifestation of disease upon exposure to *Listeria monocytogenes*. *L. monocytogenes* is seen as an opportunistic disease since most listeriosis patients have a physiological or pathological defect that affects T-cell-mediated immunity facilitating pathogen invasion (Vázquez-Boland *et al.* 2001b).

2.5.2.1 Invasion of the intestine



Once *Listeria monocytogenes* is ingested with food, it first has to cross the intestinal wall before severe symptoms of listeriosis is manifested. *L. monocytogenes* enters with the aid of p60 and internalins, which facilitates its attachment to intestinal walls. A study undertaken with rodents showed that a point of entry for *L. monocytogenes* was Peyer's patches lining the intestine, where they were found to multiply rapidly. They were then carried in macrophages or dendritic cells through the lymphatic system to mesenteric lymph nodes and then via the blood stream to the liver, spleen, placenta and central nervous system (Chen *et al.* 2000; Doyle 2001) (Figure 2.4a and 2.4b).

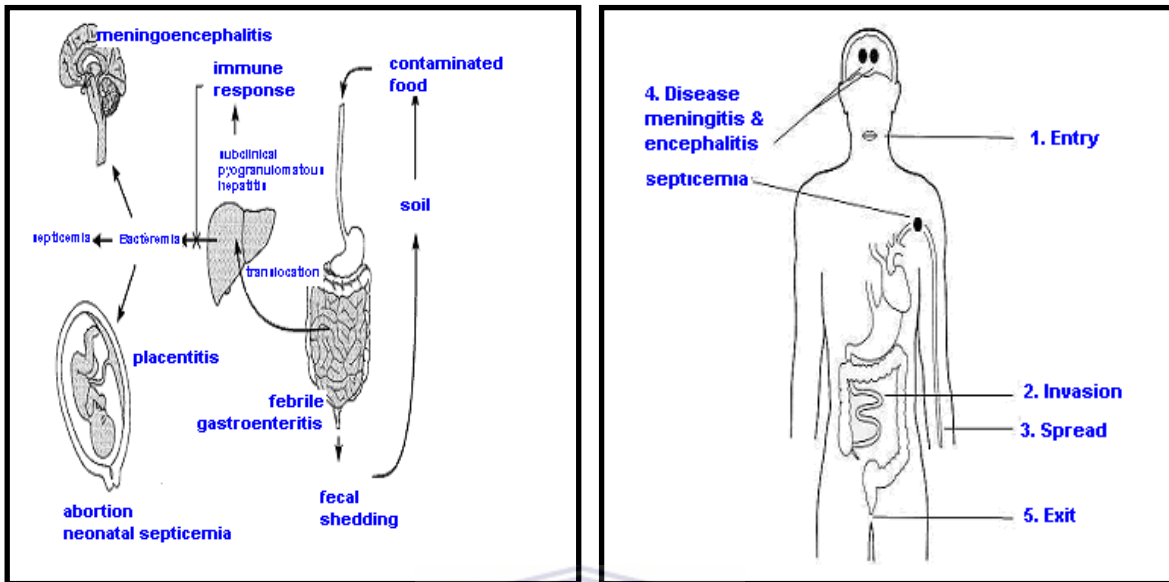


Figure 2.4a *L. monocytogenes* infection cycle. The pathogen is capable of crossing three barriers, namely the intestinal, blood-brain and placental barrier (adapted from Vázquez-Boland *et al.* 2001b)

Figure 2.4b The pathogenicity of *L. monocytogenes*. Infection results in two main clinical manifestations, namely sepsis and meningitis (adapted from Hof, Baron's Medical Microbiology, Miscellaneous pathogenic bacteria).

2.5.2.2 Invasion of the liver

As noted above, the *Listeria monocytogenes* that cross the intestinal barrier are carried by the lymph or blood to the mesenteric lymph nodes, the spleen, and the liver. If an adequate immune response is not elicited in the liver, then *L. monocytogenes* will proliferate which will facilitate its invasion/spread to the bloodstream (Vázquez-Boland *et al.* 2001b) and cause septicaemia.

2.5.2.3 Invasion of the placenta

Pregnant women represent a high risk-group for *Listeria monocytogenes* infection (Abram *et al.* 2003). *L. monocytogenes* targets and crosses the placental barrier in pregnant women. The crossing of the placental barrier leads to serious fetal infections, fetal death, miscarriages, premature births and neonatal infections (Longhi *et al.* 2003; Todar 2003; Schett *et al.* 2005). The interaction between a protein from *L. monocytogenes*, internalin, and its host's cell receptor, E-cadherin (a transmembrane protein expressed by epithelial cells), has been shown to facilitate entry to the human placental barrier (Lecuit *et al.* 2004; Dominguez-Bernal *et al.* 2006). Macrophages in the body that contain *Listeria* and enter the placenta infect endothelial cells and then the fetus resulting in premature labour or death of the fetus, otherwise sepsis or meningitis will result if the infant is infected while passing through the birth canal (Doyle 2001). Serovar analysis from patients identified serotype 4b as being the most prevalent in pregnancy-associated cases (Doyle 2001).

2.5.2.4 Invasion of the brain

Listeria monocytogenes is able to invade endothelial cells and is known to cause meningitis and encephalitis in infected individuals resulting in a high mortality rate (Schuchat *et al.* 1991; Greiffenberg *et al.* 1998). A study undertaken by Greiffenberg *et al.* (2000) showed that invasion of the human brain microvascular endothelial cells

(HBMEC) with *L. monocytogenes* was dependant on the *inlB* gene. An electron micrograph showed that *L. monocytogenes* invades the HBMEC and that intracellular multiplication, movement and production of bacterium containing protrusions accompany it. The fact that *L. monocytogenes* can invade HBMEC, illustrates that it is able to cross the blood-brain barrier (Figure 2.5).

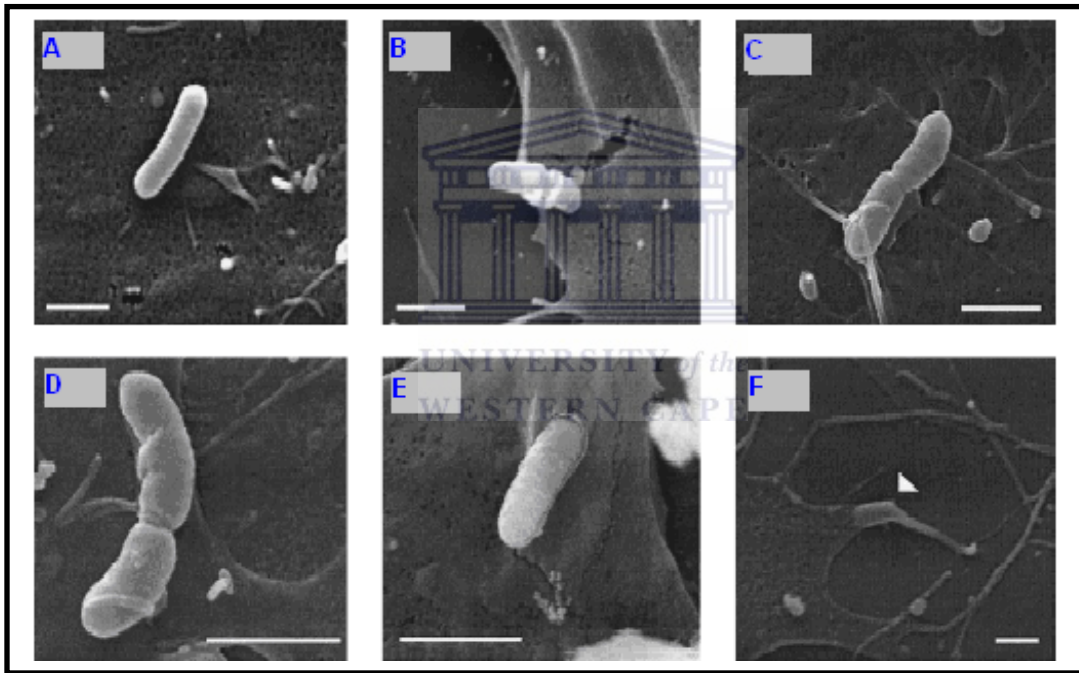


Figure 2.5 An electron micrograph showing a 35 minute postinfection of HBMEC with *L. monocytogenes*. **A** *L. monocytogenes* on the cell surface or; **B, C, D** in contact with microvilli; **E** rarely was *L. monocytogenes* seen in the process of invasion or; **F** already taken up by the HBMEC (arrowhead) (adapted from Greiffenberg *et al.* 2000)

2.6 DETECTION METHODS FOR *LISTERIA*

2.6.1 Conventional (traditional) methods

Conventional methods for microbial detection require the target organism to grow and form colonies on a specific growth medium. These methods, although fairly cheap, are labour intensive with regard to media preparation and recording the results as well as time consuming (a time period of 5-7 days to complete) (Norton and Batt 1999; Choi and Hong 2003; Gouws and Liedemann 2005). Conventional methods have also showed to be unreliable especially for thermally injured or stressed organisms (Norton and Batt 1999). Conventional methods for detecting micro-organisms require several stages namely dilution, pre-enrichment, selective enrichment broth, selective plating and biological and/or serological tests (Lantz 1998; Purwati *et al.* 2001; Neamatallah *et al.* 2003).

2.6.1.1 *Diluting a sample*

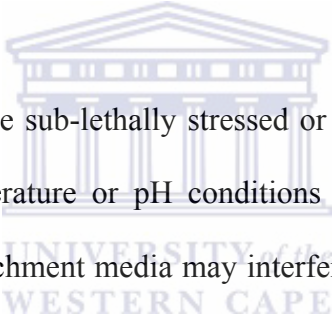
Homogenizing a food sample such as diluting the material generates a large volume of material. The distance between the inhibitors and target molecule is increased, thereby reducing the chance of interference of the inhibitor with the target (Tsai and Olen 1992). Diluting a sample has shown to be effective when aiming to reduce the effect of inhibitors on the PCR reaction (Fredericks and Relman 1998). In a study undertaken by Fredericks and Relman (1998), the inhibitory effect of sodium polyanetholesulfonate

(SPS), an additive to blood culture medium, could only be overcome by diluting the samples. Where low numbers of the target cell is present, diluting a sample will only reduce the bacterial numbers even more, therefore an enrichment step should be performed before a dilution is carried out to ensure that detection of the target cell is allowed.

2.6.1.2 Pre-enrichment

The recovery of bacterial pathogens from foods including raw milk, dairy products and meat can be complicated due to the presence of high numbers of indigenous microflora and other pathogens and because the pathogens of interest may be sublethally injured at the time of testing. Enrichment in nonselective (pre-enrichment) and selective media is usually required for the detection and identification of food-borne pathogens. The recovery of *Listeria* spp. from food requires the use of selective enrichment media such as *Listeria* enrichment broth (LEB), containing antibiotics that are inhibitory to competitive microorganisms. Much effort has been employed to find an enrichment media and protocol for *L. monocytogenes* and other *Listeria* spp. isolation. An ideal enrichment medium would promote the recovery and proliferation of *L. monocytogenes* and *Listeria* spp. over other microflora (Cocolin *et al.* 2002). Pre-enrichment or primary enrichment broths generally have smaller amounts of selective agents, which promote the recovery of injured/stressed cells (Beumer and Hazeleger 2003). With conventional detection methods, false positives may arise as a result of other *Listeria* spp. being

present with *L. monocytogenes*. Overgrowth of *Listeria* spp. may also result during the enrichment procedure resulting in *L. innocua* out-competing *L. monocytogenes* (Norton *et al.* 2001; Willis *et al.* 2006) and mimicking the appearance of *L. monocytogenes* on a culture medium. For this reason further testing to differentiate between species needs to be employed. The function of the enrichment steps prior to the PCR reaction increases the number of target cells (after 4 h the bacteria should double in numbers) and secondly it facilitates the PCR reaction by reducing false negative results (Ericsson and Stålhandske 1997; Duffy *et al.* 2001).



Listeria monocytogenes may be sub-lethally stressed or injured when present in a food product due to extreme temperature or pH conditions and in certain cases, selective agents present in selective enrichment media may interfere with the repair of these cells, since selective media contain agents which select for healthy target organisms. These cells, which are then undetected, can recover and grow during the storage of food (Pearson and Marth 1990; Wu and Fung 2001; Yuste *et al.* 2003; Rijpens and Herman 2004; Gasanov *et al.* 2005). Since food samples and enrichment media can be inhibitory to the PCR reaction, it may be necessary to subculture in a non-selective medium prior to the PCR reaction (Gouws and Liedemann 2005). In some cases the enumeration of *L. monocytogenes* from food products was greater when a short, non-selective enrichment step was employed rather than a complete, long selective enrichment step (Rijpens and Herman 2004). In a study undertaken by Rijpens and Herman (2004), results showed that when a non-selective enrichment step was employed (using buffered peptone water

(BPW)), the recovery rate of *L. monocytogenes* increased within some cheeses. This non-selective enrichment step was either employed for 5 or 24 h followed by a shorter selective enrichment step, compared to one long selective enrichment step.

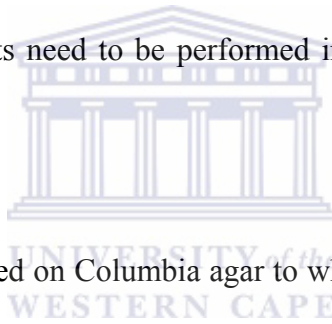
2.6.1.3 Selective enrichment broth

By using Fraser broth, optimum growth conditions are created for *Listeria* due to the high nutrient content and the large buffer capacity, which enhances cell growth and repair. The growth of accompanying bacteria is largely inhibited by selective agents - lithium chloride, nalidixic acid and acriflavine hydrochloride. The glucose esculin is cleaved by β -D-glucosidase into esculetin and glucose. The esculetin then forms an olive-green to black complex with the iron (III) ions resulting in a blackening of the broth indicating the presence of *Listeria* spp. (Gasarov *et al.* 2005; Oxoid manual (<http://www.oxoid.com/uk/index.asp>))

2.6.1.4 Selective plating

Listeria cells tend to grow slowly and are outgrown by other competitors. For this reason selective agents such as acriflavin and nalidixic acid have been added to selective agar or enrichment broths to facilitate the proliferation of *Listeria* while suppressing competing microflora in food and environmental samples (Gasarov *et al.* 2005). Selective plating represses the growth of some organisms so others will grow. This is achieved by adding

inhibitors or imposing certain conditions. Oxford and PALCAM agar are selective for *Listeria* based on esculin hydrolysis; however it does not differentiate *L. monocytogenes* from the rest of the species (Neamatallah *et al.* 2003; Marrakchi *et al.* 2005). In a study undertaken by Marrakchi *et al.* (2005), Oxford agar was shown to be more effective than PALCAM agar for the isolation of *L. monocytogenes* from marine samples; however a limitation that it does not distinguish *L. monocytogenes* from the other spp. of its genus exists, especially important for the recovery of *L. monocytogenes* from food products. It is therefore evident that these conventional detection methods is time consuming and laborious since subsequent tests need to be performed in order to differentiate between species.



Oxford agar formulation is based on Columbia agar to which lithium chloride, acriflavin, colistin sulfate, sefotetan, cycloheximide and fosfomycin have been added. These ingredients suppress the growth of gram - negative bacteria and the greater part of gram - positive bacteria. β -D-glucosidase hydrolyses esculin into esculetin and forms a black complex with iron (III) ions. Therefore, *L. monocytogenes* produces grey-green coloured colonies with a black halo (Merck manual 1996; Willis *et al.* 2006). Some other organisms that are able to utilize esculin example, *Bacillus* spp. and *Enterococcus* may mimic the appearance of *Listeria* spp. therefore further tests may need to be performed in order to confirm presumptive results (Gasarov *et al.* 2005).

PALCAM (Polymyxin Acriflavin Lithium chloride Ceftazidime Aesculin Mannitol) agar provides selective isolation of *Listeria* while at the same time inhibiting the gram-negative and most of the gram-positive accompanying bacteria. Mannitol and a pH indicator, phenol red, have been added to differentiate mannitol-fermenting strains from *Listeria* based on mannitol fermentation. Mannitol fermentation is demonstrated by a colour change in the colony and/or the surrounding medium from red to grey to yellow due to the production of acidic end-products (Difco manual 1998). The media comprises polymyxin, acriflavin, ceftacidim and lithium chloride. *L. monocytogenes* breaks down the esculin in the medium to esculetin and glucose. Esculetin forms an olive-green to black complex with iron (III) ions, which stains the colonies of *L. monocytogenes* (Merck manual 1996). Colonies of *Listeria* appear grey-green with a black precipitate after inoculation and incubation at 35°C for 24-48 h (Allerberger 2003).

Compared to selective agars, non-selective agars allow for the growth of non-injured and sub-lethally stressed organisms but allow no differentiation within the population (Wu and Fung 2001).

2.6.1.5 Gram Stain and API- *Listeria*

For further confirmation and identification, microscopy can be employed to differentiate between a Gram-positive and Gram-negative organism. *Listeria* are Gram positive, slim, short and rod-shaped. The API-*Listeria* (BioMérieux), which includes ten tests, relies on

biochemical analysis that is based on the fermentation of a range of sugars (Allerberger 2003; Beumer and Hazeleger 2003). Since selective plating does not distinguish between species of the genus *Listeria*, hemolytic activity (lysing of red blood cells) is the marker used to distinguish *Listeria monocytogenes* from *L. innocua*, which tends to outgrow and mimic the appearance of *L. monocytogenes* on culture media. Of the six species, is only *L. monocytogenes*, *L. seeligeri* and *L. ivanovii* hemolytic. Hemolytic activity can be detected on horse or sheep blood containing agar plates; however the API-Listeria test, which includes a 'DIM' test, differentiates *L. monocytogenes* from *L. innocua* based on the presence of acrylamidase without considering hemolytic activity (Allerberger 2003). Acrylamidase is present in *L. innocua* strains but absent in *L. monocytogenes* (Billie *et al.* 1992). The API-Listeria consists of the following ten tests. DIM tests for the presence or absence of acrylamidase, ESC tests for the hydrolysis of esculin, α -MAN tests for the presence of α -mannosidase, DARL tests for acid production from D-arabitol, XYL tests for acid production from D-xylose, RHA tests for acid production from L-rhamnose, MDG tests for acid production from α -methyl-D-glucoside, RIB tests for acid production from D-ribose, G1P tests for acid production from glucose-1-phosphate and TAG tests for acid production from D-tagatose.

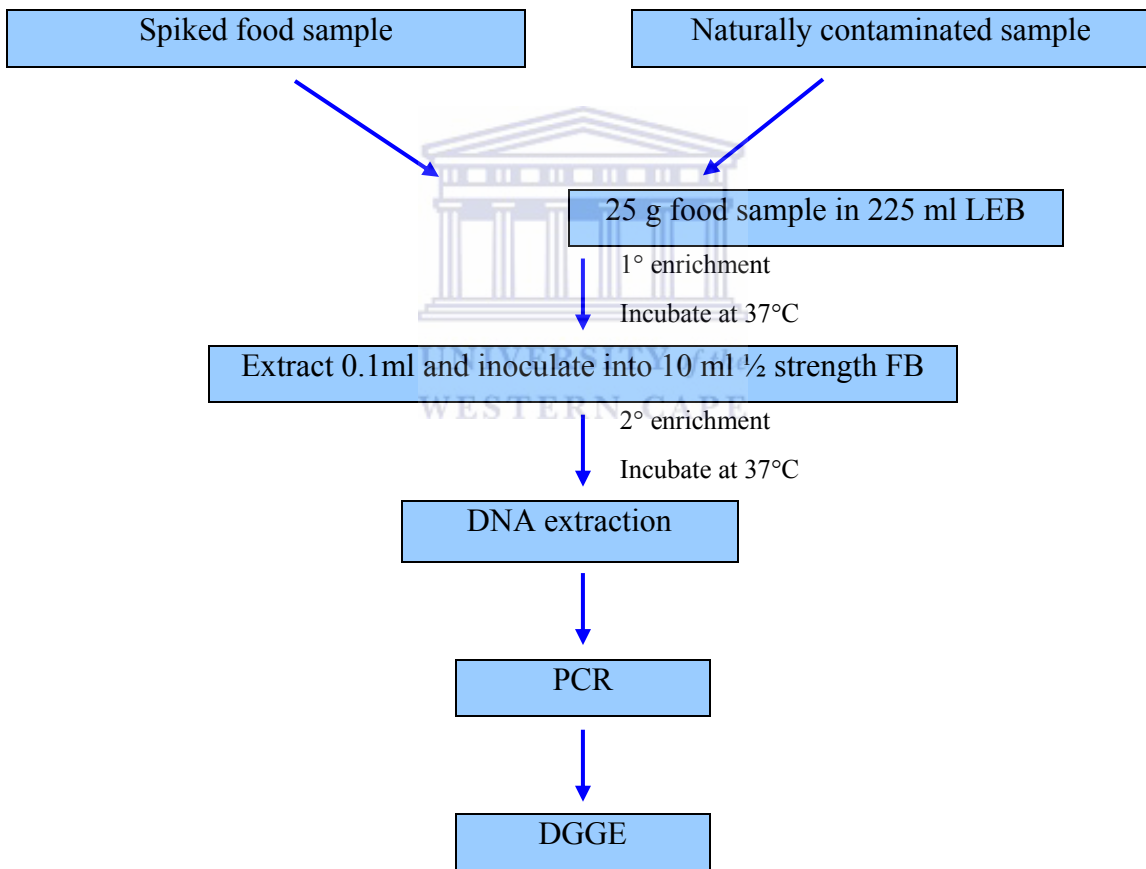
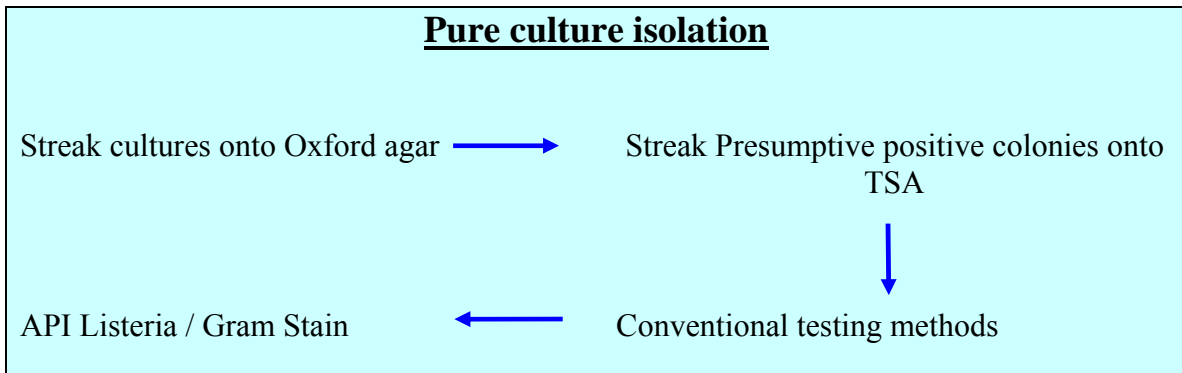


Figure 2.6 Generic method for the isolation and detection of *L. monocytogenes* in food products.

2.6.2 MOLECULAR BASED DETECTION METHODS

2.6.2.1 Detection of microorganisms by the PCR reaction

Polymerase Chain Reaction is a technique employed for the rapid detection of microorganisms within food products (Kim *et al.* 2001; Holko *et al.* 2002; Gouws and Liedemann 2005). It is a very specific and sensitive technique, which was discovered by Kary M. Mullis (Mullis 1990). PCR is a molecular based method, known for overcoming the limitations of conventional methods for the detection of microorganisms (Al-Soud 2000). The detection of microorganisms by the PCR reaction is divided into four stages namely, sample collection, sample preparation, amplification and detection (Figure 2.7) (Lantz 1998). As a result of the low concentration of pathogens in some complex food samples as well as the presence of PCR inhibitors that will reduce or block DNA amplification, samples have to be treated (pre-PCR treatment) as to increase bacterial numbers to facilitate detection by PCR thereby enhancing DNA recovery. A limitation of the PCR reaction is that various inhibitors present in biological samples could limit its potential. These inhibitors tend to interfere with the cell lysis step, inactivate the DNA polymerase or interfere with the nucleic acids rendering a false negative result in the PCR reaction (Al-Soud and Rådström 2000). The sample preparation step is considered to be the most important and necessary step in determining the PCR results since it reduces/eliminates problems associated with PCR inhibitors and determining the sensitivity of the PCR reaction (Lantz 1998). The PCR reaction, unlike conventional

detection methods, is able to eliminate false positives and detect the presence of *L. monocytogenes* in food products (Gouws and Liedemann 2005).

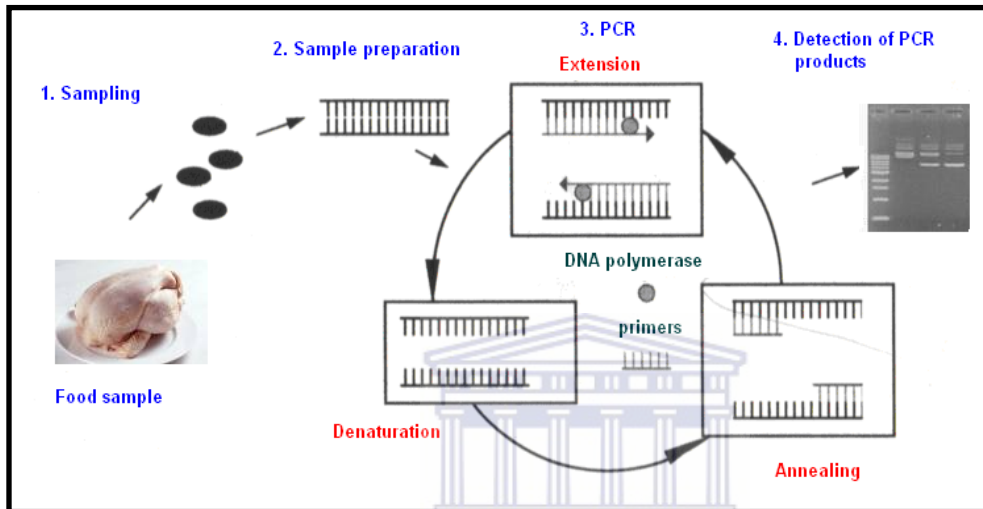


Figure 2.7 The detection of micro-organisms by PCR. This process is usually divided into 4 stages, namely sample collection, sample preparation, DNA amplification and detection (Adapted from Lantz 1998).

2.6.2.2 PCR facilitators

The basic components of a PCR mixture are template DNA, pH buffer, magnesium ions, deoxynucleotides, primers and thermostable DNA polymerase. The amount of template in the reaction strongly influences performance in the PCR reaction. The recommended amount of template for the standard PCR reaction is 1-10 ng for bacterial DNA and 0.1-1 ng for plasmid DNA (Roche PCR Application Manual 1999).

2.6.2.3 PCR inhibitors

Specific food components have been shown to inhibit the PCR reaction and the identification of these components can assist when trying to increase the sensitivity of the PCR reaction in order to detect the pathogen present (Kim *et al.* 2000). Certain components have been identified as PCR inhibitors such as hemoglobin, haemin, immunoglobulin G, lactoferrin in blood, myoglobin in muscle, bile salts, complex polysaccharides, proteinases in faeces, anticoagulants, collagen, sodium polyanetholesulfonate (SPS) (a common additive to blood culture medium which tends to co-purify with the DNA), substances in milk and soft cheeses (Akane *et al.* 1994; Fredericks and Relman 1998; Kim *et al.* 2000; Stöcher *et al.* 2003). For this reason, it is therefore necessary to design pre-PCR treatments in order to reduce the effects of PCR inhibitors and thereby maximize DNA recovery when trying to isolate *L. monocytogenes* from food products.

2.6.2.4 Internal amplification control

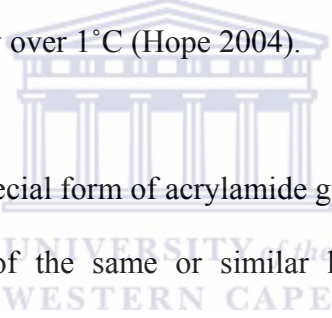
A problem that may arise with PCR is that false-negative results may arise as a result of the PCR reaction being completely inhibited or there may be a reduction in the PCR product yield due to failure in the amplification reactions (Al-Soud 2000; Stöcher *et al.* 2003). Different strategies have been developed to detect such failure which may be a result of the DNA extraction method, the pre-PCR treatments employed, inhibition of the

DNA polymerase, malfunction of the PCR machine or the incubation of the PCR mixture (Hoorfar *et al.* 2003; Wieczorek and Osek 2004). One of the best ways to detect failure of PCR amplification reactions is to include a non-target DNA sequence, an internal amplification control (IAC), because it is amplified together with the target sequence under the same conditions (Al-Soud 2000; Stöcher *et al.* 2003; Hoorfar *et al.* 2004; Rodríguez-Lázaro *et al.* 2004a). There are two ways to amplify the IAC, either 2 primer sets are used in multiplex PCR, one pair for the target sequence and the other pair for the non-target sequence, or one primer pair is used to amplify the target sequence and the non-target sequence (Sachadyn and Kur 1998). Where one primer set is used, it may be designed so that the 5' overhanging ends of the forward and reverse IAC primer are identical to the primer sequence for the target (diagnostic) sequence and 3' ends are complementary to the pre-determined region of the non-target sequence selected. The region of the non-target sequence to be amplified would have to differ in size compared to the amplicon of the target DNA in order for their DNA fragments to separate and be visualized clearly and precisely when Agarose gel electrophoresis is used.

2.6.2.5 Denaturing gradient gel electrophoresis

In order to distinguish between different species of a genus, the DGGE technique can be employed. The DGGE technique separates PCR amplicons of the same size but different sequences (Ercolini 2004), which is a powerful tool for mutation detection (Hayes *et al.* 1999) and very useful in epidemiological investigations. The two strands of the DNA

molecule melt or separate when heat or a chemical denaturant is applied. The temperature at which the double strand melts is influenced by two factors namely, GC rich domains (covalent - 3 bonds) which melt at a higher temperature compared to AT rich domains (covalent - 2 bonds) which denatures more easily and secondly, the attraction between neighboring bases of the same strand namely, stacking interactions. The degree of stacking is determined by the order of bases on the strand. Therefore, depending on the nucleotide sequence, the molecules which differ by only one nucleotide will have several melting domains. A single base change may affect the stacking interaction enough to alter the melting temperature (T_m) by over 1°C (Hope 2004).



The DGGE technique uses a special form of acrylamide gels that can separate small (200-700 bp) genomic fragments of the same or similar length but with different base composition. The gel is poured in a gradient increasing in denaturing strength, provided with formamide and urea, in the direction of the electrophoretic run (100% denaturing strength consists of 40% formamide and 7M urea) (Hayes *et al.* 1999; Ercolini 2004). The melting behaviour of a DNA fragment determines its migration pattern in the gel. The mobility of the DNA molecule changes at the concentration at which the DNA strands with a low melting domain separate resulting in a partially single stranded molecule (Hope 2004). A partially denatured fragment moves much more slowly or becomes entangled in the gel matrix resulting in no movement through the polyacrylamide gel compared to a single- or double-stranded fragment. When DNA is loaded into the denaturing gel, the double-stranded DNA molecules become partially

melted and their mobility decreases within the gel. However, if the double-stranded DNA molecules become completely melted into single strands, their mobility increases (Figure 2.8). A good resolution results when the DNA molecules do not completely separate (Ercolini 2004). To prevent total denaturation of the DNA molecule a GC rich sequence, a GC-clamp with a high melting domain, is attached to one primer before PCR amplification (Hayes *et al.* 1999; Chang Bioscience 2004).

A study undertaken by Cocolin *et al.* (2002) showed that nine strains identified as *Listeria monocytogenes* by conventional methods proved to be *L. innocua* by the DGGE method (direct identification in food samples) and that it was also possible to differentiate between serotypes.

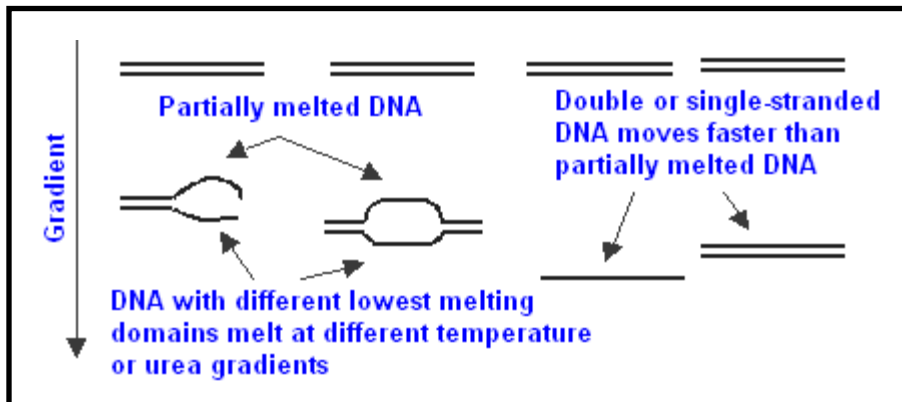
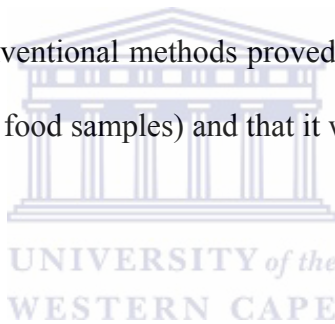
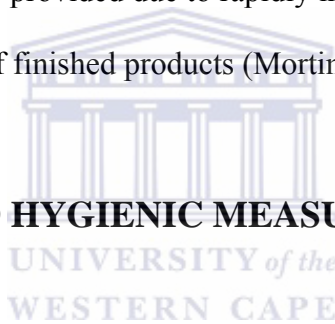


Figure 2.8 Illustration of the movement of DNA in denaturing gradient gels (Adapted from Chang Bioscience 2004).

2.7 MICROBIAL FOOD-BORNE HAZARDS

A hazard is a physical (e.g. glass, wood), chemical (e.g. cleaning material) or biological (e.g. bacteria) property, which may cause the food to be unsafe for human consumption. Micro-organisms such as *Salmonella enteritidis*, *Listeria monocytogenes*, *Campylobacter jejuni* and verotoxigenic *Escherichia coli* have stressed food safety systems resulting in legislations being implemented in some countries for improved consumer protection. Food safety assurance has to be provided due to rapidly increasing global sourcing of raw materials and the distribution of finished products (Mortimore and Wallace 1994).



2.8 HACCP AS A GOOD HYGIENIC MEASURE

Food safety is when all conditions in the food chain from production to consumption have been assessed to ensure that no health risks exist when consuming the food product. The safety of the food is compromised when these conditions have not been met (Beumer and Hazeleger 2003). Hazard Analysis and Critical Control Point (HACCP) is a system that is currently being used in the food industry by the government regulatory agencies because it has shown to be effective in preventing the occurrence of food-borne biological, chemical and physical hazards. It is essentially a system of control, which is based on the prevention of problems. HACCP looks for hazards, or what could go wrong, to make a product unsafe for human consumption. Thereafter, control and management systems are implemented to ensure that the product is safe and cannot cause harm to the

consumer (Mortimore and Wallace 1994). The implementation of HACCP to control or prevent *Listeria monocytogenes* contamination in the food-processing environment has been regarded as an effective measure to control or prevent listeriosis outbreaks and should be implemented by food producers and food preparers (Choi and Hong 2003; Beumer and Hazeleger 2003).

Where raw milk or pasteurized milk is used to manufacture cheese, it is vital to look at the whole process so that critical control points (CCP) can be identified (Leite *et al.* 2006). The HACCP system to control the presence of *L. monocytogenes* in pasteurized milk focuses on the selection of raw milk as well as controlling the processing, packaging, distribution and storage conditions (Xanthiakos *et al.* 2006). Since *L. monocytogenes* is commonly isolated from products in the fish-processing environment such as cold smoked fish, HACCP programmes are vital for the seafood industry (Norton *et al.* 2001). The spread of bacteria to food products should be prevented in the food processing environment by implementing HACCP as a system of control. The critical points need to be monitored regularly to prevent *L. monocytogenes* contamination (Rórvik 2000).

The HACCP system was originally developed as a safety system for manned space programmes in the United States to ensure food safety for the astronauts, but it was discovered that a high level of food safety assurance was required, which resulted in an effective HACCP system being implemented (Mortimore and Wallace 1994).

2.8.1 The seven principles of HACCP

These include conducting a hazard analysis, identifying the CCP, establishing critical limits, CCP monitoring requirements, corrective actions, record keeping and verification procedures. These HACCP principles have international acceptance. They outline how to establish, implement and maintain a HACCP plan for the system under investigation (National Advisory Committee on microbiological criteria for foods 1997).

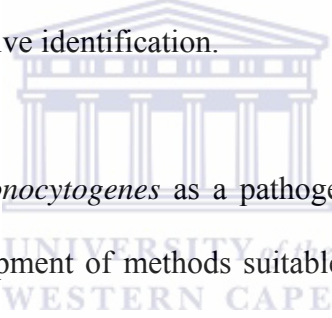
2.9 CONCLUSION



Listeria monocytogenes is a serious threat to the safety of food and its presence in food is an indicator of poor hygiene. Since regulatory agencies have issued a zero tolerance ruling for the presence of *L. monocytogenes* in certain food products, effective detection, isolation and confirmation methods are crucial. Detection methods have to be sensitive enough to detect low *L. monocytogenes* levels and to allow the enumeration of stressed or injured cells because of their ability to transmit disease through food products.

The detection of non-pathogenic *Listeria* spp. is considered an indicator for the presence of smaller numbers of the pathogenic *L. monocytogenes* spp. that may be present in food samples, but may go undetected due to the overgrowth of the other species. For this reason, microbiological methods that allow detection and differentiation of all *Listeria* spp. within food products are advantageous.

Due to the low concentration of pathogens and the presence of PCR inhibitors in food samples that is known to interfere with DNA amplification, different pre-PCR treatments are designed in order to increase DNA recovery and reduce inhibition. Once a successful pre-PCR treatment is designed, the PCR technique is employed to detect the presence of pathogens in a sample. Hence, the sample preparation must carefully be selected and treated in order to use the specificity and speed of the PCR reaction to its full potential. The PCR technique is employed for its rapid and reliable detection of microorganisms compared to the conventional methods of detection, which is time consuming, labor intensive and provide presumptive identification.



Since the recognition of *L. monocytogenes* as a pathogen causing listeriosis, there has been an advance in the development of methods suitable for isolation and detection. A limitation that may arise when using selective media for *L. monocytogenes* detection is that differentiation between the species is not always possible. Therefore, more advanced, rapid methods such as the PCR reaction and DGGE method is employed which is able to differentiate at the species level. By optimizing the PCR protocol, improvements in the quality control of food products will result as well as an increase in knowledge on these pathogens and in which food products they prevail. By selecting methods such as DGGE analysis for pathogen detection, fast and easy identification of all species belonging to the genus *Listeria* is allowed. This identification is more conclusive compared to conventional detection methods, which are less reliable.

CHAPTER 3

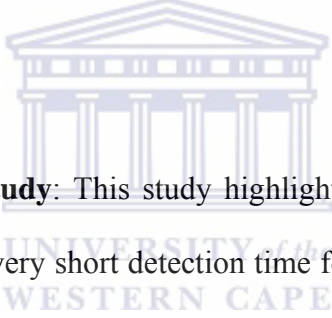
Evaluation of the factors affecting the sensitivity of *Listeria monocytogenes* isolation and PCR detection in food products

3.1 ABSTRACT

Aim: This study investigated the performance of various sample preparation methods and polymerase chain reaction (PCR) facilitators for the detection of *Listeria monocytogenes* in artificially and naturally contaminated food samples in order to obtain a reproducible set of conditions that would enhance DNA amplification and augment the specificity and sensitivity of the protocol.

Materials and Methods: Artificially contaminated food samples (25 g), camembert cheese, hake, minced meat and ostrich meat were pre-enriched in *Listeria* enrichment broth for 5 h followed by a 17 h secondary enrichment in ½ Fraser broth. DNA extracts were subjected to PCR amplification and the PCR products were electrophoresed on agarose gels. A cheese sample contaminated with *L. monocytogenes* was obtained from a cheese manufacturer and this protocol was used to confirm its specificity and sensitivity for the detection of *L. monocytogenes* in a naturally contaminated food product.

Conclusion: The results demonstrated that with a 5 h enrichment in *Listeria* enrichment broth, the PCR assay could detect as few as 7-9 cfu ml⁻¹ and 1 cfu 25 g⁻¹ in artificially contaminated food samples. Where 5 h enrichment in buffered peptone water (BPW) was used instead of *Listeria* enrichment broth (LEB), the PCR assay was compromised as 10²-10³ cfu ml⁻¹ was the number of organisms that needed to be present for PCR amplification to occur. When the efficacy of DNA polymerase was evaluated, *Taq* DNA polymerase and *Tth* DNA polymerase were found to be equally effective in their ability to overcome inhibition in food products, rendering an isolation and detection protocol that was rapid with a very high sensitivity.



Significance and impact of study: This study highlighted a very robust, efficient and reproducible procedure with a very short detection time for the isolation and detection of exceptionally low numbers of *L. monocytogenes* in food products; which is much desired in the food industry.

3.2 INTRODUCTION

Listeria monocytogenes contamination is a problem in the food production environment. This is due to the fact that *L. monocytogenes* can survive and grow at refrigeration and freezer temperatures, which are conditions used for food production and storage in order to prevent spoilage of food products (Rudi *et al.* 2005).

The detection and isolation of *L. monocytogenes* from food products is challenging due to the presence of other organisms within the food product. In this respect the isolation method is critical and must allow for the recovery and detection of injured cells, keeping in mind that rapid and reliable detection methods for pathogen recovery are desirable in the food industry (Pearson and Marth 1990; Marrakchi *et al.* 2005). Food producers, distributors and public health authorities have great interest in rapid methods that are reliable, inexpensive, sensitive and specific (Beumer and Hazeleger 2003).

For a test to be approved by regulatory agencies, it must be able to detect one *Listeria* organism 25 g⁻¹ (1 cfu 25 g⁻¹) of food product. Enrichment protocols have to be specific enough, usually allowing growth of 10⁴-10⁵ cfu ml⁻¹ in order for this sensitivity to be reached (Gasnov *et al.* 2005).

Conventional selective methods, including selective enrichment for *Listeria*, may take up to several weeks and does not always allow for the enumeration of stressed/injured cells.

It is therefore important for the enrichment and isolation methods for the recovery of stressed/injured cells to be carefully selected in order to control *Listeria* associated food-borne disease (Gasnov *et al.* 2005).

Selective agar, such as Oxford and PALCAM, does not distinguish *L. monocytogenes* from the other species of the genus and for this reason further testing needs to be performed in order to confirm presumptive positive results (Willis *et al.* 2006).

The PCR reaction is considered a reliable and reproducible technique for the identification of *Listeria* spp. It is able to differentiate *L. monocytogenes* from the rest of the species of the genus by using primers that are specific to the *hly* listeriolysin O gene. An advantage that molecular techniques, such as the PCR reaction, has over conventional detection methods is that characterization is based within the genome as opposed to identification being based on other expression factors such as esculin hydrolysis on selective agars (Gasnov *et al.* 2005). Phenotypic characteristics that are expressed when using conventional detection methods may be unreliable and difficult to interpret (Gouws and Liedemann 2005). Molecular methods are becoming increasingly popular since they are more accurate, sensitive and specific, making it more reliable.

A major challenge with regard to testing food for bacterial pathogens has always been the interference of molecular tests by inhibiting food components. Studies revealed that the sensitivity on the PCR detection limit was compromised when food samples were spiked

with bacterial cells as opposed to the PCR detection limit obtained for pure cultures (Aznar and Alarcón 2003).

Polymerase is an enzyme that is involved in the synthesis of DNA. Thermostable DNA polymerase is a key component in the PCR reaction. *Taq* DNA polymerase is a validated lab polymerase that is used in most PCR assays. It is isolated from the bacterium *Thermus aquaticus* whereas *Tth* DNA polymerase is isolated from the thermophilic eubacterium *Thermus thermophilus*. These enzymes display thermal stability and can withstand denaturation at high temperatures (Löfström *et al.* 2004).

The function of various thermostable DNA polymerases have shown to be inhibited differently by PCR inhibitors which suggests that the appropriate thermostable DNA polymerase should be used to overcome the effect of inhibitors and thereby amplify the pathogen present in the food product (Al-Soud and Rådström 2000; Kim *et al.* 2000; Lúbeck *et al.* 2003). In a study undertaken by Lúbeck *et al.* (2003) to demonstrate the effect of PCR inhibitory substances in chicken samples on 3 different enzymes, it was found that *Tth* DNA polymerase was more resistant to the inhibitors compared to *Taq* DNA polymerase and DyNAzyme and for this reason *Tth* DNA polymerase was selected as the appropriate polymerase for internal amplification design (IAC) design.

3.3 MATERIALS AND METHODS

3.3.1 Reference strains

Glycerol stocks of *Listeria monocytogenes* ScottA (UWC L1) and *Listeria monocytogenes* NCTC 4855 were resuscitated by resuspending 100 µl of the culture into 10 ml Tryptone Soy Broth (Oxoid) and incubated overnight at 37°C (Norton and Batt 1999; Smith *et al.* 2001).



3.3.2 Sample preparation

The overnight culture was diluted 10-fold in ½ strength Fraser broth (½ FB), covering a dilution range from 10^0 - 10^{-7} . The food samples namely; camembert cheese, hake, ostrich meat and minced meat (25 g), were prepared using sterilized instruments and spiked with 100 µl of overnight culture (10^0 - 10^{-7}) and thereafter homogenized in 225 ml of enrichment broth using a Stomacher 400 laboratory blender (Seward Ltd). To test the efficacy of various pre-enrichment broths in their ability to dilute inhibitors and improve the sensitivity of the PCR reaction, the food samples were pre-enriched in BPW, LEB supplemented with *Listeria* selective supplement UVM1 (Oxford formulation) and ½ FB, all supplied by Oxoid and prepared according to the manufacturer's instructions. Pre-enrichment in LEB produced the best results and was used in subsequent experiments. All food samples were incubated at 37°C for 5 h. Thereafter, 0.1 ml was extracted and

inoculated into 10 ml $\frac{1}{2}$ FB (Oxoid). This suspension was put on a shaker (114 rpm) at 37°C for 17 h.

3.3.3 DNA isolation from spiked food samples

A method modified by Agarsborg *et al.* (1997) was used for DNA isolation. Following the 17 h secondary enrichment, a 2.0 ml aliquot culture was transferred to a 2.0 ml eppendorf tube. Cultures were centrifuged at 9000 x g for 10 min. The pellet was resuspended in 400 μ l sterile distilled water to which 400 μ l 2% Triton-X-100 (BDH Chemicals Ltd) was added and the contents mixed. This suspension was left at room temperature for 10 min, thereafter incubated at 100°C for 10 min and then centrifuged at 9000 x g for 4 min. The supernatant was transferred to a sterile eppendorf tube and 1 μ l of this crude cell lysate was used for PCR amplification.

3.3.4 PCR amplification

PCR amplification was specific for a 730 bp product of the *hly* virulence gene of *L. monocytogenes* (Blaise and Phillippe 1995). Careful optimization of constituent quantities as well as thermal cycling parameters took place. For a 25 μ l reaction, the mixture contained: 1 X PCR buffer (Celtic Molecular Diagnostics, Bioline), MgCl₂ (final concentration 5mM with *Taq* and *Biotaq* DNA polymerase and 1.5mM with *Tth* DNA polymerase) (Whitehead Scientific (Promega), Celtic Molecular Diagnostics (Bioline)

and Roche Diagnostic respectively), dNTP's (final concentration 200 µM) (Roche Diagnostic), LmonoF and LmonoR primers (Blaise and Phillippe 1995) (final concentration 0.3 µM each) (Whitehead Scientific, IDT), 1U DNA polymerase (*Tth*, *Biotaq* and *Taq* – Roche Diagnostic, Celtic Molecular Diagnostics (Bioline) and Whitehead Scientific (Promega) respectively) and 1 µl template DNA (10^0 - 10^{-7}). The sequence for LmonoF and LmonoR is illustrated in Table 3.1.

Amplification was carried out in a thermal cycler GeneAmp[®] PCR system 2700 (Applied Biosystems) with the following optimized programme: Initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 40 s and extension at 72°C for 1 min with a final extension step at 72°C for 2 min. The PCR products (7 µl) underwent electrophoresis on a 1% agarose D-1 LE gel (Whitehead Scientific) and were visualized by staining with ethidium bromide. The amplified PCR products were viewed using the AlphaImager[®] HP system (AlphaInnotech Corporation). Gel pictures were acquired using the AlphaEase FC[™] software version 4.0.0.

Table 3.1 Sequence of the primer set specific for the amplification of the *hly* gene of *L. monocytogenes* (Blaise and Phillippe 1995).

Description	Primer sequence	Amplicon size
Primer set specific for the amplification of the <i>hly</i> <i>L. monocytogenes</i> gene.	LmonoF: 5' - CAT TAG TGG AAA GAT GGA ATG - 3'	730 bp
	LmonoR: 5' - GTA TCC TCC AGA GTG ATC GA - 3'	

3.4 RESULTS AND DISCUSSION

In order to determine the effect of various pre-enrichment broths on the sensitivity of the PCR assay; BPW, LEB and ½ FB were compared in order to determine which method of pre-enrichment produced the best result. The enrichment step was incorporated to increase the number of pathogens to a detectable concentration as well as to dilute inhibitors present in the food samples. When food samples were pre-enriched in a non-selective broth (BPW) and selective *Listeria* broth (½ FB) for 5 h and treated accordingly (section 3.3.3 and 3.3.4), the results proved that pre-enrichment in ½ FB was more inhibitory to the sensitivity of the PCR reaction compared to BPW. Enrichment in BPW enhanced the sensitivity of the PCR reaction as 10^2 cfu ml⁻¹ was the number of organisms that could be detected by PCR reaction compared to a detection limit attained at 10^3 cfu ml⁻¹ for samples enriched in ½ FB (Figure 3.1).

Pre-enrichment in LEB was then employed to assess its effect on the specificity and sensitivity of the PCR reaction. Compared to all three enrichment broths tested, LEB proved to be the most reliable and specific for its application in sample preparation methods prior to PCR analysis. Pre-enrichment in LEB provided the most consistent results and was most effective in diluting the inhibitors in the food sample. For this reason pre-enrichment in LEB for 5 h was used in subsequent experiments. Following the 5 h enrichment in LEB, a secondary enrichment in ½ FB for 17 h was performed. This sample preparation method which comprised a short primary selective enrichment step

followed by a longer secondary selective enrichment step proved to be very effective and specific for the proliferation of *L. monocytogenes* over other inhibiting microflora. Enrichment in nonselective media and selective media is usually required for the detection and identification of food-borne pathogens. Pre-enrichment or primary enrichment broths generally have smaller amounts of selective agents, which promote the recovery of injured/stressed cells (Beumer and Hazeleger 2003). Surprisingly, the sample preparation method that was most effective in this study made use of LEB and ½ FB, these are selective broths for both primary and secondary enrichments.



Figure 3.1 Comparison of two different enrichment broths for the detection of *L. monocytogenes* from spiked ostrich meat samples. Samples were pre-enriched in ½ FB (lane 2-6) and BPW (lane 8-12). **Lane 1:** 100 bp DNA ladder (Promega); **lane 2:** 9×10^6 cfu ml⁻¹; **lane 3:** 9×10^5 cfu ml⁻¹; **lane 4:** 9×10^4 cfu ml⁻¹; **lane 5:** 9×10^3 cfu ml⁻¹; **lane 6:** 9×10^2 cfu ml⁻¹ (no amplification); **lane 7:** negative control (water); **lane 8:** 5×10^6 cfu ml⁻¹; **lane 9:** 5×10^5 cfu ml⁻¹; **lane 10:** 5×10^4 cfu ml⁻¹; **lane 11:** 5×10^3 cfu ml⁻¹; **lane 12:** 5×10^2 cfu ml⁻¹; **lane 13:** negative control (water).

PCR inhibitors in food samples may be difficult to overcome when using certain DNA polymerases (Kim *et al.* 2000). For this reason various DNA polymerases were tested in order to determine which one was the most efficient in overcoming the effect of inhibitors present in the food samples tested (Figure 3.2). When evaluating and comparing DNA polymerase, namely *Tth* (Roche Diagnostic), *Taq* (Whitehead Scientific, Promega) and *Biotaq* (Celtic Molecular Diagnostics, Bioline); no significant differences were obtained when either polymerase was employed in the PCR reaction; the specificity of the assay was shown to be unaffected by changing the DNA polymerase. However, it should be noted that after careful optimization of constituent quantities of PCR reagents and facilitators; optimal conditions were only created when magnesium chloride ($MgCl_2$) was used at a final concentration of 5mM with *Taq* and *Biotaq* DNA polymerase and 1.5mM with *Tth* DNA polymerase. When incorporated into the PCR reaction at a lower or higher concentration; assay sensitivity was compromised. Although the same detection limit was attained when using all three enzymes in the PCR reaction; generally the performance of Bioline *Biotaq* and *Tth* DNA polymerase was more reliable compared to Promega *Taq* DNA polymerase, when employed in a series of reactions. Only when sample preparation methods and PCR reagents were optimized for PCR analysis, was Promega *Taq* DNA polymerase more proficient in overcoming the effect of inhibitors and thereby function more effectively.

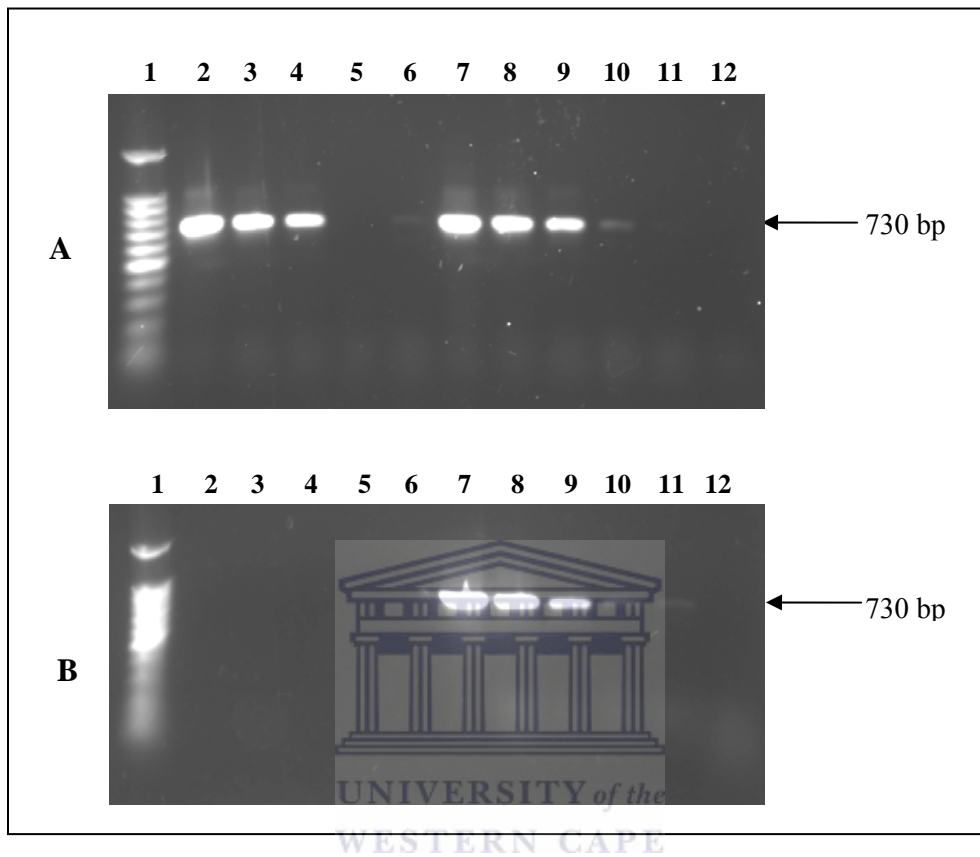


Figure 3.2 Evaluation of Bioline *biotaq*, Promega *Taq* and Roche's *Tth* to determine one most efficient in overcoming the effect of inhibitors. Ostrich meat samples were spiked with *L. monocytogenes* ScottA, pre-enriched in BPW, subjected to a 10-fold dilution series and treated as in section 3.3. **Lane 1:** 100 bp DNA ladder (Promega); **A - lanes 2-6:** Bioline *Biotaq* DNA polymerase; **lane 2:** 6×10^6 cfu ml⁻¹; **lane 3:** 6×10^5 cfu ml⁻¹; **lane 4:** 6×10^4 cfu ml⁻¹; **lane 5:** 6×10^3 cfu ml⁻¹; **lane 6:** 6×10^2 cfu ml⁻¹; **lane 7-11:** Promega *Taq* DNA polymerase; **lane 7:** 6×10^6 cfu ml⁻¹; **lane 8:** 6×10^5 cfu ml⁻¹; **lane 9:** 6×10^4 cfu ml⁻¹; **lane 10:** 6×10^3 cfu ml⁻¹; **lane 11:** 6×10^2 cfu ml⁻¹; **lane 12:** negative control (water); **B - lanes 2-6:** *Tth* DNA polymerase with 3.75 mM MgCl₂; **lanes 7-11:** *Tth* DNA polymerase with 1.5 mM MgCl₂; **lane 7:** 6×10^6 cfu ml⁻¹; **lane 8:** 6×10^5 cfu ml⁻¹; **lane 9:** 6×10^4 cfu ml⁻¹; **lane 10:** 6×10^3 cfu ml⁻¹; **lane 11:** 6×10^2 cfu ml⁻¹; **lane 12:** negative control (water).

The sample preparation method and optimized PCR protocol were now tested on food products to determine how robust and specific this method was for the detection and isolation of *L. monocytogenes* from food samples and more importantly the detection limit that could be determined by using this protocol. Camembert cheese, hake, minced meat and ostrich meat samples were spiked with *L. monocytogenes* ScottA and *L. monocytogenes* 4855 and subjected to sample preparation methods and the PCR reaction. As few as 8 cfu ml⁻¹ and 7 cfu ml⁻¹ *L. monocytogenes* was detected by the PCR reaction, when ostrich meat samples were spiked with *L. monocytogenes* ScottA and *L. monocytogenes* 4855 respectively (Figure 3.3 and 3.4). It has been reported in literature that at least 10³ cfu ml⁻¹ needs to be present in order for detection by the PCR reaction to occur (Aznar and Alarcón 2003); whereas other studies specify that 10⁴-10⁵ cfu ml⁻¹ was the detection limit for PCR analysis (Guo *et al.* 2000; Zhou and Jiao 2005). The optimized protocol used in this study provided a much higher sensitivity and specificity for the isolation and identification of *L. monocytogenes* since as few as 7 cfu ml⁻¹ was detected by the PCR reaction. This method was then tested on camembert cheese, hake and minced meat samples and consistent results were obtained each time. When these food samples were spiked with *L. monocytogenes* ScottA, a minimum of 9 cfu ml⁻¹ for camembert cheese and hake and 8 cfu ml⁻¹ for minced meat was detected by the PCR reaction (Figure 3.5).

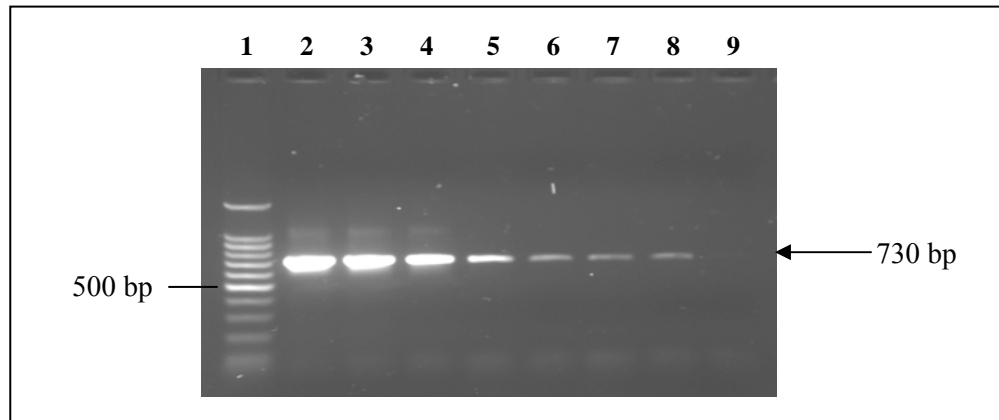


Figure 3.3 The minimum number of *L. monocytogenes* ScottA in ostrich meat detectable by the PCR reaction. Ostrich meat samples were spiked with *L. monocytogenes* ScottA, pre-enriched in LEB, diluted 10-fold and subjected to DNA extraction and PCR. The cfu ml⁻¹ was then calculated. **Lane 1:** 100 bp DNA ladder (Promega); **lane 2:** 8x10⁶ cfu ml⁻¹; **lane 3:** 8x10⁵ cfu ml⁻¹; **lane 4:** 8x10⁴ cfu ml⁻¹; **lane 5:** 8x10³ cfu ml⁻¹; **lane 6:** 8x10² cfu ml⁻¹; **lane 7:** 8x10¹ cfu ml⁻¹; **lane 8:** 8 cfu ml⁻¹; **lane 9:** negative control (water).

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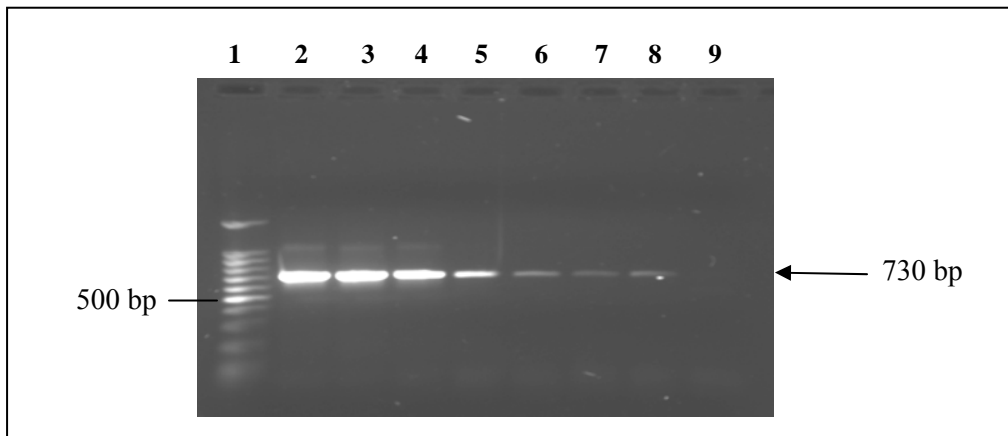


Figure 3.4 The minimum number of *L. monocytogenes* NCTC 4855 in ostrich meat detectable by the PCR reaction. Ostrich meat samples were spiked with *L. monocytogenes* NCTC 4855 and prepared as described in figure 3.3. **Lane 1:** 100 bp DNA ladder (Promega); **lane 2:** 7x10⁶ cfu ml⁻¹; **lane 3:** 7x10⁵ cfu ml⁻¹; **lane 4:** 7x10⁴ cfu ml⁻¹; **lane 5:** 7x10³ cfu ml⁻¹; **lane 6:** 7x10² cfu ml⁻¹; **lane 7:** 7x10¹ cfu ml⁻¹; **lane 8:** 7 cfu ml⁻¹; **lane 9:** negative control (water).

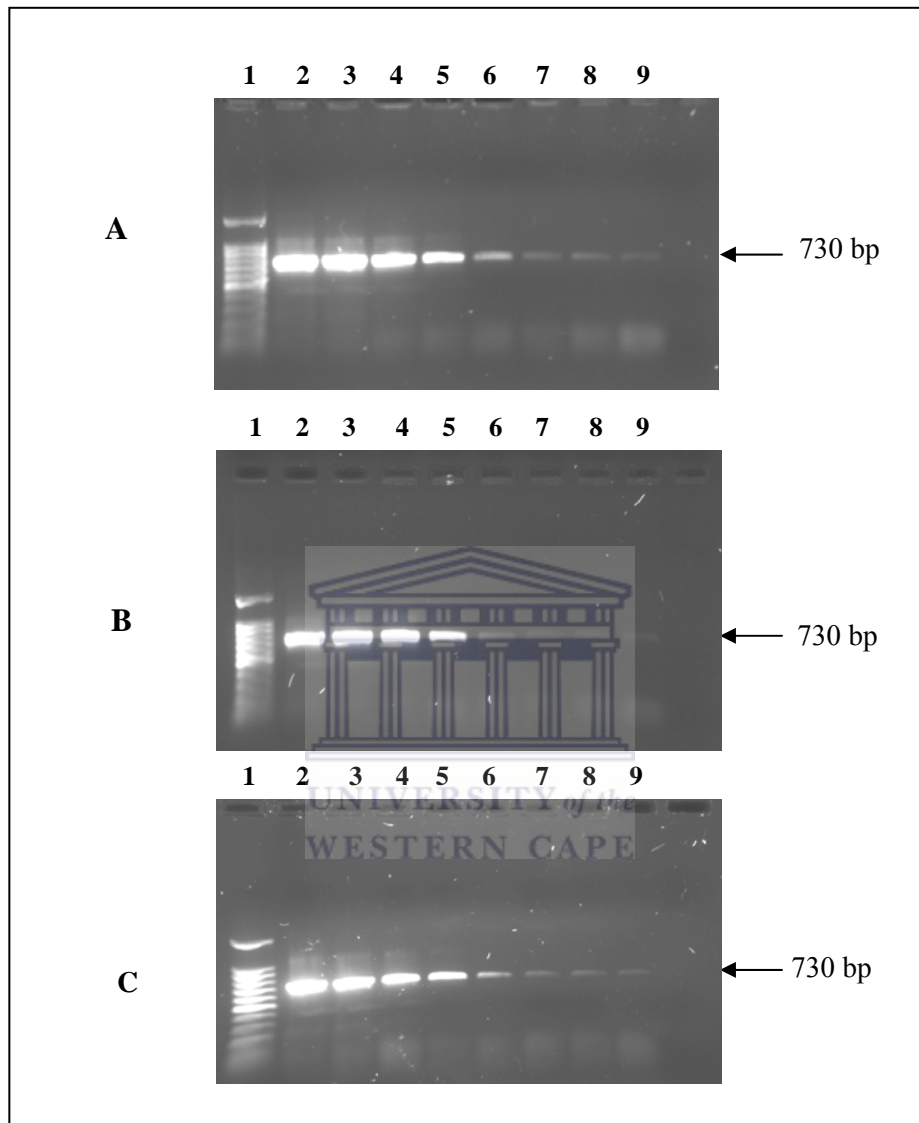


Figure 3.5 The assessment of the minimum number of *Listeria monocytogenes* ScottA in cheese, hake and minced meat that was detected by the PCR reaction. Food samples (A) camembert cheese (B) hake fish (C) minced meat; were all spiked with 100 μ l of *Listeria monocytogenes* ScottA, pre-enriched in LEB and subjected to a 10-fold dilution series. **Lane 1:** 100 bp DNA ladder (Promega); **A - lane 2:** 9×10^7 cfu ml⁻¹; **lane 3:** 9×10^6 cfu ml⁻¹; **lane 4:** 9×10^5 cfu ml⁻¹; **lane 5:** 9×10^4 cfu ml⁻¹; **lane 6:** 9×10^3 cfu ml⁻¹; **lane 7:** 9×10^2 cfu ml⁻¹; **lane 8:** 9×10^1 cfu ml⁻¹; **lane 9:** 9 cfu ml⁻¹; **B - lane 2:** 9×10^7 cfu ml⁻¹; **lane 3:** 9×10^6 cfu ml⁻¹; **lane 4:** 9×10^5 cfu ml⁻¹; **lane 5:** 9×10^4 cfu ml⁻¹; **lane 6:** 9×10^3 cfu ml⁻¹; **lane 7:** 9×10^2 cfu ml⁻¹; **lane 8:** 9×10^1 cfu ml⁻¹; **lane 9:** 9 cfu ml⁻¹;

C - lane 2: 8×10^7 cfu ml⁻¹; **lane 3:** 8×10^6 cfu ml⁻¹; **lane 4:** 8×10^5 cfu ml⁻¹; **lane 5:** 8×10^4 cfu ml⁻¹; **lane 6:** 8×10^3 cfu ml⁻¹; **lane 7:** 8×10^2 cfu ml⁻¹; **lane 8:** 8×10^1 cfu ml⁻¹; **lane 9:** 8 cfu ml⁻¹

To determine the PCR detection limit of *L. monocytogenes* in food samples; camembert cheese and ostrich meat were spiked with a known concentration of *L. monocytogenes* ScottA and the cfu g⁻¹ was then calculated. The optimized protocol designed and applied in this study resulted in a minimum of 7 cfu ml⁻¹ being detected by the PCR reaction. The sensitivity of this method was confirmed as it was calculated that as few as 1 cfu in 25 gram of food sample (1 cfu 25 g⁻¹) was the detection limit for *L. monocytogenes* in both camembert cheese samples and ostrich meat (Figure 3.6 and 3.7).

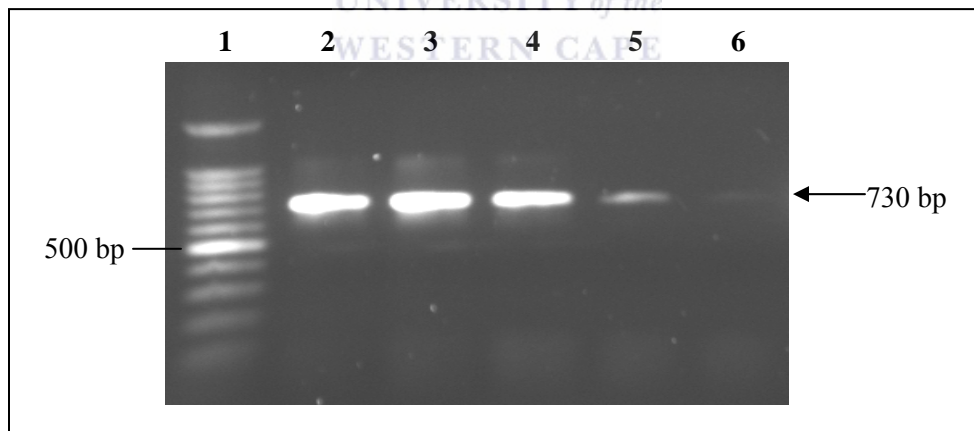


Figure 3.6 Determination of the detection limit of *L. monocytogenes* ScottA within spiked ostrich meat samples. **Lane 1:** 100 bp DNA ladder (Promega); **lane 2:** 1×10^4 cfu 25 g⁻¹; **lane 3:** 1×10^3 cfu 25 g⁻¹; **lane 4:** 1×10^2 cfu 25 g⁻¹; **lane 5:** 1×10^1 cfu 25 g⁻¹; **lane 6:** 1 cfu 25 g⁻¹

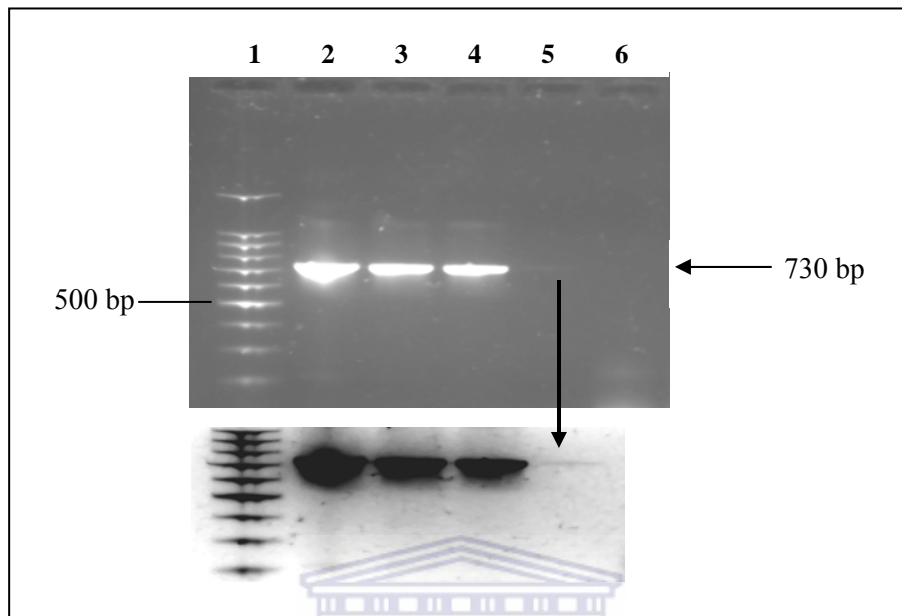


Figure 3.7 Determination of the detection limit of *L. monocytogenes* ScottA within spiked camembert cheese samples. **Lane 1:** 100 bp DNA ladder (Promega); **lane 2:** 1×10^3 cfu 25 g^{-1} ; **lane 3:** 1×10^2 cfu 25 g^{-1} ; **lane 4:** 1×10^1 cfu 25 g^{-1} ; **lane 5:** 1 cfu 25 g^{-1} ; **lane 6:** negative control (water)

Various processes have been implemented by industries to reduce the number of microorganisms in food products in order to ensure their overall safety. One such process is ultraviolet (UV) radiation; a cold pasteurization process that is an alternative approach to thermal pasteurization. During thermal pasteurization, excessive heat may cause protein denaturation and loss of vitamin and flavour compounds (Lado and Yousef 2002). The UV radiation process is a cold process that does not produce any undesirable by-products or chemical residues and economically it is more feasible. It functions by

intercalating with the bacterial cells DNA, thereby disrupting cell function. When UV light is absorbed by the DNA, pyrimidine dimers are formed between nucleotides resulting in double stranded DNA molecules becoming fused; leading to the disruption of cell function (Giese and Darby 2000).

Camembert cheese samples contaminated with *L. monocytogenes* was obtained from a cheese manufacturer. One cheese sample was prepared from milk that had undergone a UV treatment process to reduce the number of spoilage organisms (Figure 3.8).

The protocol employed in this study was used to confirm its specificity and sensitivity for the detection of *L. monocytogenes* in a naturally contaminated food product. The method proved robust and sensitive enough to detect *L. monocytogenes* in the naturally contaminated cheese sample; given the fact that organisms contaminating food products are generally in a state of injury or stress due to unfavourable conditions they are subjected to (Rijpens and Herman 2004). The fact that as few as 7 cfu ml⁻¹ *L. monocytogenes* was detectable by the PCR reaction in this study just enhanced the prospect of detecting exceptionally low numbers of *L. monocytogenes* in naturally contaminated food products.

No explanation can be provided for the non-specific band between 500-600 bp. It seems to be more pronounced in the cheese sample where the milk that was used to produce the cheese had undergone a UV treatment process. The size of the non-specific band, 500-

600 bp, has no correlation to the internal amplification control (IAC) construct designed in chapter 4, considering that the IAC 555 bp construct was only obtained when the DNA of pUC19 was included in the PCR reaction and absent when only the DNA of *L. monocytogenes* was included in the PCR reaction. The fact that *L. monocytogenes* was detected in the cheese samples made from UV treated and heat treated milk may signify that contamination of the cheese product more than likely occurred in the post-processing environment and not as a result of the milk. This experiment was repeated twice and the same result was produced. The DNA fragment was not sequenced, as all that was required was a confirmation to the presumption that the cheese sample had *L. monocytogenes* contamination.

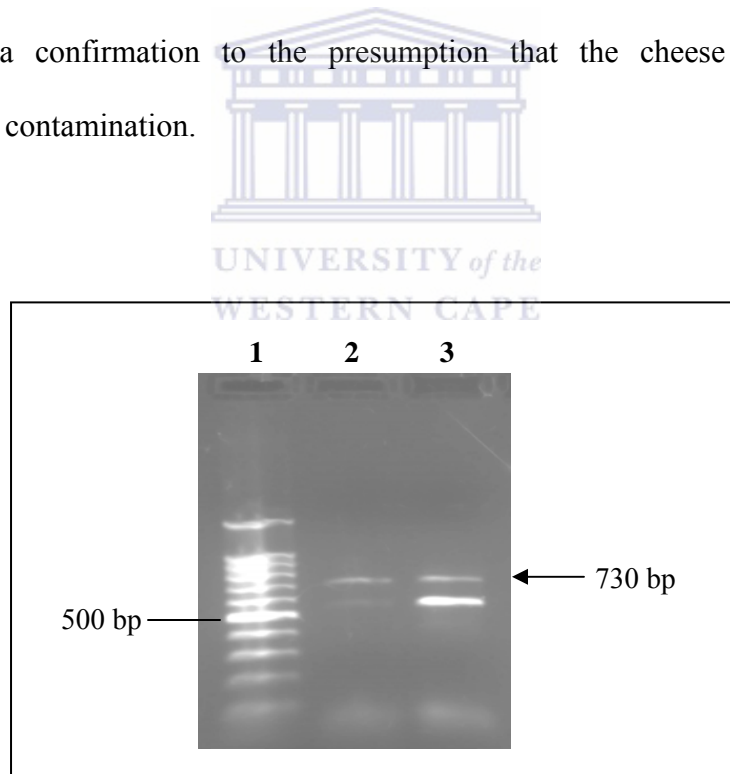


Figure 3.8 *Listeria monocytogenes* isolated and detected from a naturally contaminated camembert cheese sample. **Lane 1:** 100 bp DNA ladder (Promega); **lane 2:** a camembert cheese sample manufactured from milk that had undergone heat treatment; **lane 3:** a camembert cheese sample manufactured from milk that had undergone a UV treatment process.

3.5 CONCLUSION

Listeria monocytogenes is an important food-borne pathogen and is widely tested for in food, environmental and clinical samples. The PCR reaction targeted the *hly* gene specific to *L. monocytogenes*.

The methods proposed in this study allowed for the detection of exceedingly low numbers of *L. monocytogenes* within 26 h by the PCR reaction. As few as 7-9 cfu ml⁻¹ were detectable in camembert cheese, hake, minced meat and ostrich meat; contrary to the specification cited in many literature material that a minimum of 10³ cfu ml⁻¹ needs to be present for PCR amplification to occur. The methods implemented in this study gave a much lower *L. monocytogenes* detection limit than what is specified in current literature. Rapid and sensitive methods for detecting *L. monocytogenes* are in great demand in order to assure product safety; therefore the results of this study will have a huge impact in the food industries.

To create optimal conditions for *L. monocytogenes* isolation and detection, several parameters affecting the sensitivity for PCR detection were evaluated to find a sample preparation method and PCR procedure for the routine detection of *L. monocytogenes* in food products. Factors such as sample preparation methods, incubation times, DNA extraction methods and PCR constituents were all considered. When constructing this protocol, special thought had to be given to stressed or injured cells that may go

undetected. It can be concluded that a combination of pre-enrichment in Listeria enrichment broth, secondary enrichment in ½ Fraser broth, the Triton-X-100 DNA extraction method and PCR using the optimized protocol listed resulted in a very robust, specific and efficient protocol which increased the recovery rate of *L. monocytogenes* in food products.

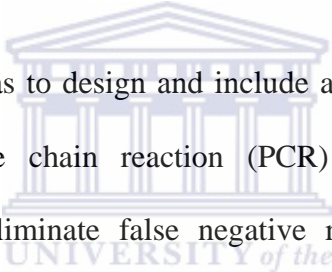
The detection limit for *L. monocytogenes* is an important parameter to consider when designing a protocol for its identification and recovery from food products. The sensitivity level of 1 cfu 25 g⁻¹ that was attained in this study fulfills the set limits on the number of *L. monocytogenes* organism in foods that are generally accepted, such as the absence in 1, 25 or 50 cfu g⁻¹ or <10² cfu 25 g⁻¹. The Department of Health, South Africa specifies zero tolerance g⁻¹ in cheese, zero tolerance 25 g⁻¹ in cooked items prior to cooling, < 10 cfu g⁻¹ in cold meal items and smoked or fermented meal items and < 100 cfu g⁻¹ in food items that require further cooking (Department of Health 2001).

The method proposed for the detection of *L. monocytogenes* has been validated in the naturally contaminated cheese sample tested and is suitable to implement in the food industry. Given the high accuracy, sensitivity and speed of the methods proposed in this study, there are no doubts that these methods for *L. monocytogenes* recovery and detection will form the basis for future routine testing of food products in the food industry.

CHAPTER 4

Internal amplification control design using multiplex PCR for the detection of *Listeria monocytogenes* in food products

4.1 ABSTRACT

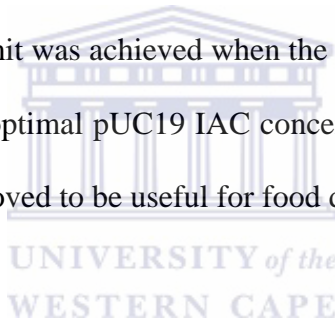


Aim: The aim of this study was to design and include an internal amplification control (IAC) within the polymerase chain reaction (PCR) to co-amplify with *Listeria monocytogenes* in order to eliminate false negative results that may arise for the identification of *L. monocytogenes* in food products. The IAC had to be incorporated into the PCR reaction without loss of specificity and sensitivity on the detection limit of *L. monocytogenes*.

Materials and methods: Ostrich meat samples and camembert cheese samples were spiked with *L. monocytogenes* ScottA (UWC L1) and pre-enriched in Listeria enrichment broth. Following pre-enrichment, the samples underwent secondary enrichment in ½ Fraser broth and were serially diluted (10-fold) in order to calculate the cfu ml⁻¹. DNA extracts were subjected to PCR analysis. A pUC19 IAC was constructed to co-amplify with *L. monocytogenes* in a multiplex PCR reaction in order to produce two amplicons; a 730 bp product characteristic of the *hly* gene belonging to *L. monocytogenes* and a 555 bp

product (including the 5' flanking regions of the IAC primer set) characteristic to a pre-determined region on the pUC19 genome.

Conclusions: A multiplex PCR system that allowed the simultaneous amplification of *L. monocytogenes* and the pUC19 IAC was successfully constructed. The optimal concentration at which pUC19 would co-amplify with *L. monocytogenes* was determined to be 0.001 pg μl^{-1} . The minimum number of organisms detected by the PCR reaction was 8 cfu ml^{-1} for *L. monocytogenes* when the pUC19 IAC was excluded from the reaction; the same detection limit was achieved when the pUC19 IAC was included in the PCR reaction. The use of an optimal pUC19 IAC concentration increased the reliability of the PCR reaction and has proved to be useful for food diagnostics.



Significance and impact of study: The pUC19 IAC provided the assurance that negative PCR results were truly negative; since a false negative PCR result is a major threat to the food industry, as zero-tolerance rulings are in place for the presence of *L. monocytogenes* in certain food products. The pUC19 IAC was incorporated into the PCR reaction without compromising the detection limit of *L. monocytogenes* and was developed and tested for use in a multiplex PCR detection system for *L. monocytogenes* in food products. This IAC-PCR test could form the basis of a robust and standardized method for the detection of *L. monocytogenes* in food products in both research and commercial laboratories.

4.2 INTRODUCTION

The PCR reaction is a molecular based method known for overcoming the limitations of conventional methods for the detection of micro-organisms (Al-Soud 2000; Lübeck *et al.* 2003) and molecular typing of food-borne pathogens (Wieczorek and Osek 2004). The transition of PCR from research laboratories to commercial laboratories has encountered difficulty as a result of the lack of international standards and validation and that the results of tests developed or published by one laboratory may be difficult to reproduce by another laboratory (Hoorfar *et al.* 2003; Lübeck *et al.* 2003). Malfunctioning thermal cyclers, PCR inhibiting substances, inhibition of DNA polymerases, non-optimized pre-PCR treatments; all of which results in false negative PCR results; contribute to the difficulty in making the transition to commercial laboratories (Betsou *et al.* 2003; Rodríguez-Lázaro *et al.* 2004a).

Some drawbacks of the PCR reaction are that false-positive or false-negative results may occur. False positives may be avoided when proper equipment and anti-contamination procedures are followed for the isolation and detection of micro-organisms (Wieczorek and Osek 2004). False positives can be eliminated by re-testing the sample (Hoorfar *et al.* 2003).

It is important to know whether PCR failure occurred or whether it was a real negative (no band or amplification) PCR result (Müller *et al.* 1998). A false-negative PCR result is

a major threat to the food industry where the PCR reaction is being used for pathogen detection; therefore internal standards or controls have to be included in the PCR reaction to avoid false-negatives (Brightwell *et al.* 1998; Jones *et al.* 2000).

A negative PCR result does not necessarily indicate that no template DNA was present in the sample. Inhibitory substances present in a sample may be the cause of a false negative PCR result. Inhibition influences the outcome of the PCR reaction by lowering or completely preventing the amplification (Lund and Madsen 2006). One of the best ways to detect failure of the PCR reaction is to include a non-target DNA sequence, an IAC, in each reaction mix because it is amplified together with the target sequence under the same conditions (Al-Soud 2000; Stöcher *et al.* 2003; Rodríguez-Lázaro *et al.* 2004a). The amplicon size of the IAC should differ in comparison to that of the target DNA and their DNA fragments can be detected by agarose gel electrophoresis (Sachadyn and Kur 1998; Wiczorek and Osek 2004).

Where no IAC is present in the PCR sample, a negative PCR result may be indicative of no target sequence being present; however the reaction could also have been inhibited by the factors listed above. However, where an IAC is added to the PCR sample and the target DNA is not amplified but the non-target IAC is, the IAC signal (band) eliminates the possibility of false-negatives. Should the IAC signal also be absent, it would indicate the PCR reaction has failed (Rosenstraus *et al.* 1998; Betsou *et al.* 2003; Hoorfar *et al.* 2003).

It is vital to optimize the concentration of the IAC. A low concentration is usually maintained to avoid competition and inhibition of the target DNA. The IAC concentration should not be too low that no IAC signal is produced; therefore optimization is important as the amplification of one product could inhibit that of the other (Sachadyn and Kur 1998; Brightwell *et al.* 1998).

In some PCR reactions the IAC and the target DNA are amplified together using the same primer pair; another approach allows two pairs of primers to be used, one being complementary to the target DNA and the other being complementary to the non-target DNA. Initially, the IAC was designed so that the same primer pair can be used to amplify both the target *L. monocytogenes* DNA and non-target pUC19 DNA generating PCR amplicons with different sizes. The IAC primer set that was designed for this study had 5' overhanging ends which were identical to the primer sequence for *L. monocytogenes* (diagnostic sequence) and 3' ends which were complementary to the pre-determined pUC19 sequence. However, this approach for IAC design resulted in non-specific amplification directing the approach towards multiplex PCR, whereby two primer sets were used for IAC design. A pre-selected pUC19 sequence was the non-target DNA (IAC) whose DNA sequence was not homologous to the *L. monocytogenes* PCR product.

This work was aimed to develop a multiplex PCR that allowed for the detection of *L. monocytogenes* in food products while including an optimized IAC concentration to

avoid false-negative PCR results. The IAC was incorporated into the PCR reaction without loss of specificity and sensitivity on the detection limit for *L. monocytogenes*.

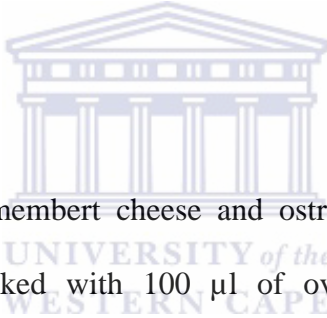


4.3 MATERIALS AND METHODS

4.3.1 Reference strains

Glycerol stocks of *Listeria monocytogenes* strain ScottA (UWC L1) was resuscitated by resuspending 100 µl of the culture into 10 ml Tryptone Soy Broth (Oxoid) and incubated overnight at 37°C (Norton and Batt 1999; Smith *et al.* 2001).

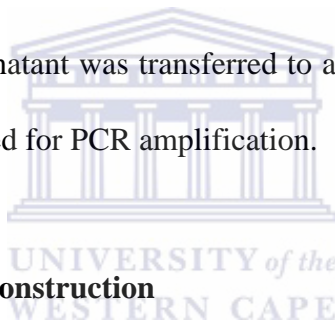
4.3.2 Sample preparation



The food samples (25 g), camembert cheese and ostrich meat, were prepared using sterilized instruments and spiked with 100 µl of overnight culture and thereafter homogenized in 225 ml of Listeria enrichment broth (LEB) (Oxoid) using a Stomacher 400 laboratory blender (Seward Ltd). All samples were incubated at 37°C for 5 h. Thereafter, 0.1 ml was extracted and inoculated into 10 ml ½ strength Fraser broth (Oxoid). This suspension was put on a shaker (114 rpm) at 37°C for 17 h. From the secondary enrichment, serial dilutions (10^0 - 10^{-6}) were performed in ½ FB and the diluted DNA was subjected to DNA extractions and PCR amplification.

4.3.3 DNA isolation from spiked food samples

A method modified by Agarsborg *et al.* (1997) was used for DNA isolation. Following the 17 h secondary enrichment, a 2.0 ml aliquot culture was transferred to a 2.0 ml eppendorf tube. Cultures were centrifuged at 9000 x g for 10 min. The pellet was resuspended in 400 µl sterile distilled water to which 400 µl 2 % Triton-X-100 (BDH Chemicals Ltd) was added and the contents mixed for 5 s. This suspension was left at room temperature for 10 min, thereafter incubated at 100°C for 10 min and centrifuged at 9000 x g for 4 min. The supernatant was transferred to a sterile eppendorf tube and 1 µl of this crude cell lysate was used for PCR amplification.



4.3.4 Primer design for IAC construction

The IAC was designed using one primer pair which would simultaneously amplify the *hly* gene of *Listeria monocytogenes* and pUC19. pUC19 was the non-target IAC that was amplified together with *L. monocytogenes* under the same conditions. The IAC primer set had 5' overhanging ends which was identical to the primer sequence for the amplification of the *hly* gene of *L. monocytogenes* (Blais and Phillippe 1995) and the 3' ends were complementary to the pUC19 DNA sequence chosen (Table 4.1). The illustration for the design of the IAC is shown in Figure 4.1. The primer sequence of the 3' end that was constructed for the amplification of a 555 bp product of the pUC19 genome was designed using the OligoAnalyzer 3.0. The genomic sequence for pUC19 was obtained from the

National Center for Biotechnology Information ([www.ncbi.nlm.nih.gov] Accession no: L09137) and BLAST-n (Basic Local Alignment Search Tool) was used to ensure that the selected oligonucleotide primers would not recognize and anneal to any other sequence but that of the pUC19 DNA. The plasmid sequence of pUC19 and genome of *L. monocytogenes*, derived from NCBI, are shown in Annexure 1 and 2. The annealing temperatures (T_m) and GC content of the pUC19 IAC primer set are listed in Table 4.2.

The IAC was designed in such a way that the 3' ends of the primer set *iacF* and *iacR* would recognize and bind to the selected DNA sequence of pUC19 resulting in flanking 5' ends, which are specific for *L. monocytogenes* DNA. The DNA polymerase was then able to synthesize and extend the template in a 5'- 3' direction, yielding the expected 555 bp product for pUC19. Similarly, the 5' ends of the same primer set would anneal to the *hly* gene of *L. monocytogenes* and synthesis by the DNA polymerase would proceed in a 5'- 3' direction, yielding a 730 bp product (Figure 4.1). However, non-specific DNA fragments were amplified when using this method of IAC construction.

Table 4.1 Construction of an IAC for the simultaneous amplification of *L. monocytogenes* and pUC19 (Sachadyn and Kur 1998; Abdulmawjood *et al.* 2002; Wiczorek and Osek 2004; Rodríguez-Lázaro *et al.* 2005). The sequences of the IAC primer set (iacF and iacR) that are identical to the *hly* gene primer set (LmonoF and LmonoR) are identified in bold. The sequence of the iacF and iacR that was designed to amplify a 555 bp region of pUC19 is underlined.

Description	Primer sequence	Amplicon size
Diagnostic primer set specific for <i>hly</i> gene of <i>L. monocytogenes</i> (Blaise and Phillippe 1995)	Forward: LmonoF 5'-CAT TAG TGG AAA GAT GGA ATG -3' Reverse: LmonoR 5'-GTA TCC TCC AGA GTG ATC GA -3'	730 bp
IAC construction for the co-amplification of <i>L. monocytogenes</i> and pUC19	Forward: iacF 5'-CAT TAG TGG AAA GAT GGA ATG <u>GCG GGT GTT GGC GGG TG</u> -3' Reverse: iacR 5'-GTA TCC TCC AGA GTG ATC GA <u>GCT GGC ACG ACA GGT TTC</u> -3'	730 bp and 555 bp

Table 4.2 Melting temperature (T_m) and GC content of primers designed for IAC.

	Primer sequence	T _m	GC content
Forward (pUC19)	5'-GCG GGT GTT GGC GGG TG-3'	60	70%
Reverse (pUC19)	5'-GCT GGC ACG ACA GGT TTC-3'	53	61%
iacF	5'-CAT TAG TGG AAA GAT GGA ATG GCG GGT GTT GGC GGG TG-3'	71	54%
iacR	5'-GTA TCC TCC AGA GTG ATC GA GCT GGC ACG ACA GGT TTC-3'	70	55%

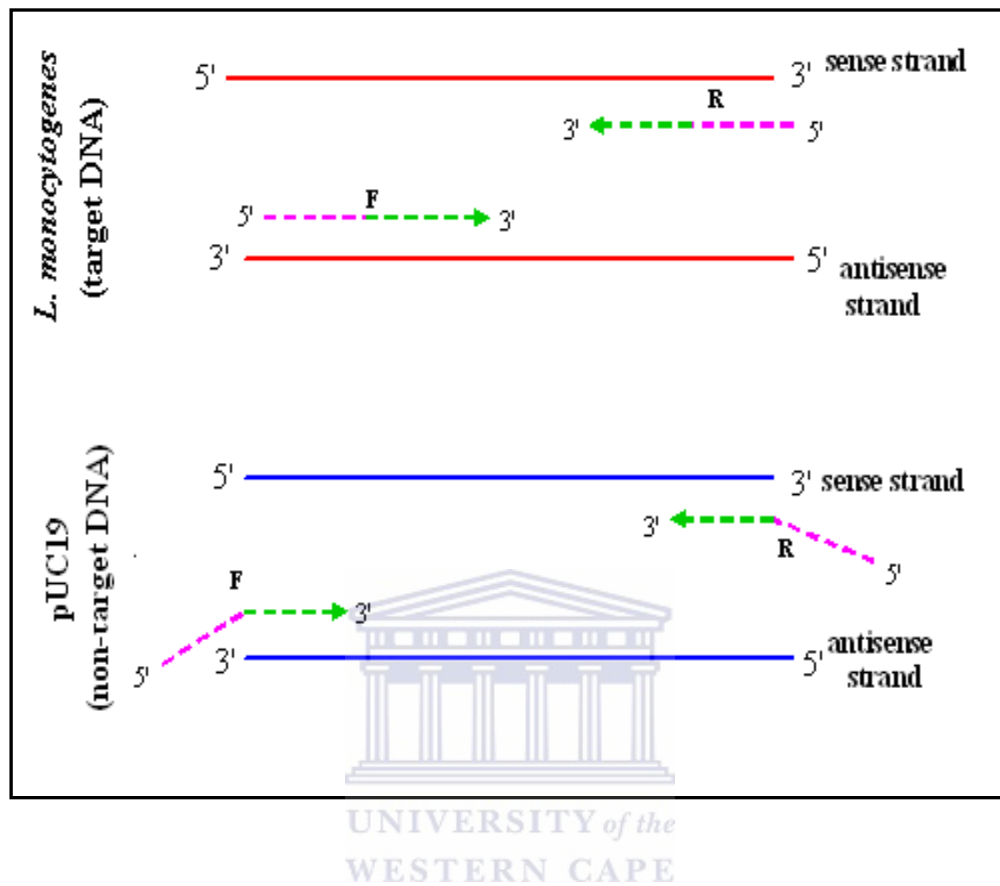


Figure 4.1 Schematic representation for the construction of the IAC (adapted from Sachadyn and Kur 1998; Abdulmawjood *et al.* 2002; Wiczorek and Osek 2004; Rodríguez-Lázaro *et al.* 2005).
 — pUC19 template DNA; — *L. monocytogenes* template DNA; - - - 3' ends of IAC primer specific for pUC19 DNA; - - - 5' ends of IAC primer specific for *L. monocytogenes* DNA; **F**-Forward primer; **R**-Reverse primer

4.3.5 Multiplex PCR

As a result of non-specific DNA being amplified when the single primer set (iacF and iacR) was used, the approach for IAC amplification was then aimed at multiplex PCR. Since the IAC primer set iacF and iacR was successful in amplifying the expected region of pUC19, but amplified *L. monocytogenes* together with other non-specific DNA, the primer set for *L. monocytogenes* was included in the PCR reaction. The primer set LmonoF and LmonoR, specific for the amplification of the *hly* gene of *L. monocytogenes*, was included together with iacF and iacR. The only change from the original IAC design was that instead of using only the IAC primer set (iacF and iacR), which was expected to amplify two products without any non-specific binding, now two primer sets were used – the diagnostic primer set for *L. monocytogenes* and the IAC primer set, which generated two amplicons, an expected 555 bp product and a 730 bp product (Table 4.2).

4.3.6 Gradient PCR

To determine the annealing temperature which was optimal for primer annealing and extension, gradient PCR was employed using the Eppendorf Mastercycler gradient (Merck). Temperature ranges of 35°-75°C were used. Initially, 55°-75°C was the range of temperatures applied for gradient PCR; however since non-specific amplification was not eliminated at these temperatures, it was decided to experiment at temperatures as low as 35°C. When the approach for IAC design was directed towards multiplex PCR, optimal

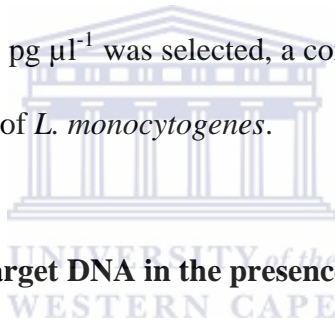
annealing temperature was observed at a temperature range of 55°-65°C and 59°C was selected as the optimal temperature at which no non-specific DNA was amplified.

4.3.7 PCR amplification for IAC construction

For a 25 µl reaction, the mixture contained: 1 X PCR buffer (final concentration) (Celtic Molecular Diagnostics, Bioline), MgCl₂ (final concentration 5mM) (Celtic Molecular Diagnostics, Bioline), dNTP's (final concentration 200 µM) (Roche Diagnostic), LmonoF and LmonoR primers (final concentration 0.3 µM each) (Whitehead Scientific IDT), iacF and iacR primers (final concentration 0.3 µM each) (Whitehead Scientific IDT), 1U *Biotaq* DNA polymerase (Celtic Molecular Diagnostics, Bioline), 1 µl template DNA (10⁰-10⁻⁶) and pUC19 (at different concentrations; refer section 4.3.8) (New England Biolabs). Amplification was carried out in a thermal cycler GeneAmp[®] PCR system 2700 (Applied Biosystems) with the following programme: Initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 59°C for 40 s, extension at 72°C for 1 min and a final extension step at 72°C for 5 min. The PCR products underwent electrophoresis on a 1% agarose D-1 LE gel (Whitehead Scientific) and visualized by staining with Ethidium Bromide. The IAC amplicon, 555 bp, is smaller than the 730 bp *hly* – specific amplicon, making distinction between the two products possible by gel electrophoresis. The amplified PCR products were viewed using the Alphaimager[®] HP system (AlphaInnotech Corporation). Gel pictures were acquired using the AlphaEase FC[™] software version 4.0.0.

4.3.8 IAC detection limit

pUC19 DNA was tested at various concentrations to determine the optimal concentration at which co-amplification with *L. monocytogenes* would occur. It had been observed that too high a concentration of the pUC19 IAC would inhibit the amplification of *L. monocytogenes*, resulting in a false negative PCR result or otherwise have an effect on the detection limit of *L. monocytogenes* by decreasing the detection limit. pUC19 was tested at a multiple range of concentrations ranging from 0.001 pg μl^{-1} to 1 pg μl^{-1} and finally a concentration of 0.001 pg μl^{-1} was selected, a concentration which resulted in no inhibition for the amplification of *L. monocytogenes*.



4.3.9 Detection limit for the target DNA in the presence of the IAC

The detection limit for the amplification of *L. monocytogenes* in the presence of the IAC was investigated. A 10-fold dilution series of *L. monocytogenes* (10^0 - 10^{-6}) was performed with pUC19 at a constant concentration of 0.001 pg μl^{-1} in order to determine the detection limit of *L. monocytogenes* by the PCR reaction. The DNA concentrations were determined using the NanoDrop^R ND-100 spectrophotometer (NanoDrop Technologies) and the bacterial cfu ml^{-1} (from dilutions in Fraser broth) was calculated by performing the spread plate technique on Tryptone soy agar (TSA) (Oxoid) and incubated at 37°C overnight. To evaluate the effect of the IAC on the detection limit of *L. monocytogenes*, the diluted DNA was amplified without the incorporation of the IAC and the results were

then compared. These experiments were repeated several times to confirm the results obtained.



4.4 RESULTS AND DISCUSSION

When the genomic DNA of only pUC19 was included in the PCR reaction, together with the IAC primer set (iacF and iacR), the expected region of pUC19 was successfully amplified, with no non-specific amplification, indicating that the IAC primer set was successful in annealing to and synthesizing the expected region of pUC19. However, when the DNA of *L. monocytogenes* was included in the PCR reaction, non-specific binding and amplification was observed resulting in a 555 bp product characteristic of pUC19 with a series of non-specific bands (Figure 4.2 and 4.3). A range of annealing temperatures were employed (35°-75°) to find the optimal annealing temperature for primer binding; however, non-specific DNA fragments were still amplified over this range of temperatures. It perceptibly appeared that there were problems with the 5' end of the primer set (iacF and iacR) annealing to the *hly* gene of *L. monocytogenes*. When the genomic DNA of pUC19 was omitted from the PCR reaction, the result was the same, non-specific DNA fragments were amplified, once again proving that the problem was more likely with the orientation of the 5' end of the IAC primer set and not due the presence of pUC19 DNA in the reaction. The formation of non-specific DNA fragments or heteroduplexes should not have occurred as the sequence of the IAC primer set, other than the 5' ends, were not homologous to the PCR target product of *L. monocytogenes* and in turn the 5' ends were not homologous to the pUC19 sequence (Annexure 3 and 4). A possible explanation for the amplification of non-specific DNA fragments when only the IAC primer set (iacF and iacR) was used may have been the orientation of the 5' end

of the primer, as noted earlier. The primer set was designed so that the 5' end would anneal to the DNA sequence of *L. monocytogenes*; however extension/synthesis by the DNA polymerase may have been inhibited due to the oligonucleotide primer sequence for pUC19 sitting on the 3' end and preventing amplification of the *L. monocytogenes* DNA sequence (Figure 4.1).



Figure 4.2 The PCR result obtained when using one primer set (*iacF* and *iacR*). Gradient PCR with an annealing temperature range of 55°C-65°C was implemented, yielding a series of non-specific bands. There clearly was an inhibitory effect on the amplification of *L. monocytogenes*. **Lane 1:** 100 bp DNA ladder (Promega); **lane 2-11:** a temperature range of 55°C-65°C increasing from left to right; **lane 2:** 55.2°C; **lane 3:** 55.7°C; **lane 4:** 56.6°C; **lane 5:** 57.8°C; **lane 6:** 59.1°C; **lane 7:** 60.5°C; **lane 8:** 61.8°C; **lane 9:** 63.1°C; **lane 10:** 64.2°C; **lane 11:** 65°C; **lane 12:** negative control (water).

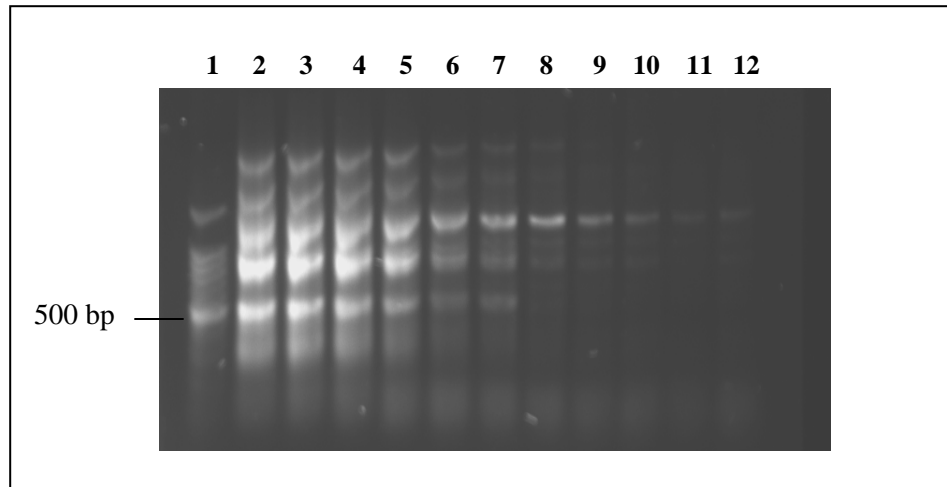


Figure 4.3 The PCR result obtained when using one primer set (iacF and iacR). Touchdown gradient PCR was employed with an annealing temperature range of 55°C-65°C decreasing by 0.5°C for 20 cycles of amplification. A temperature range of 45°-65°C was therefore covered. **Lane 1:** 100 bp DNA ladder (Promega); **lane 2-12:** touchdown gradient PCR displaying an increase in temperature from left to right (temperatures listed in Figure 4.2).

Since non-specific DNA fragments were amplified when using only the iacF and iacR primer pair, multiplex PCR using two primer sets was employed, to eliminate the occurrence of non-specific binding. When the diagnostic primer set (LmonoF and LmonoR) was incorporated in the PCR reaction together with the IAC primer set, the expected product sizes were obtained without any non-specifics (Figure 4.4). For future application, the 5' ends of the IAC primer set (iacF and iacR), specific for *L. monocytogenes* amplification, could be deleted as these were the oligonucleotides added initially, when the approach for IAC design was intended to use one primer pair for the amplification of both the *hly L. monocytogenes* gene and pUC19 IAC. This multiplex

PCR approach was very successful as the expected product sizes of 555 bp (IAC control) and 730 bp (*L. monocytogenes*) were obtained. Where either pUC19 or *L. monocytogenes* was absent from the PCR mix; the primer sets would still amplify the DNA that was present without non-specific binding and amplification.

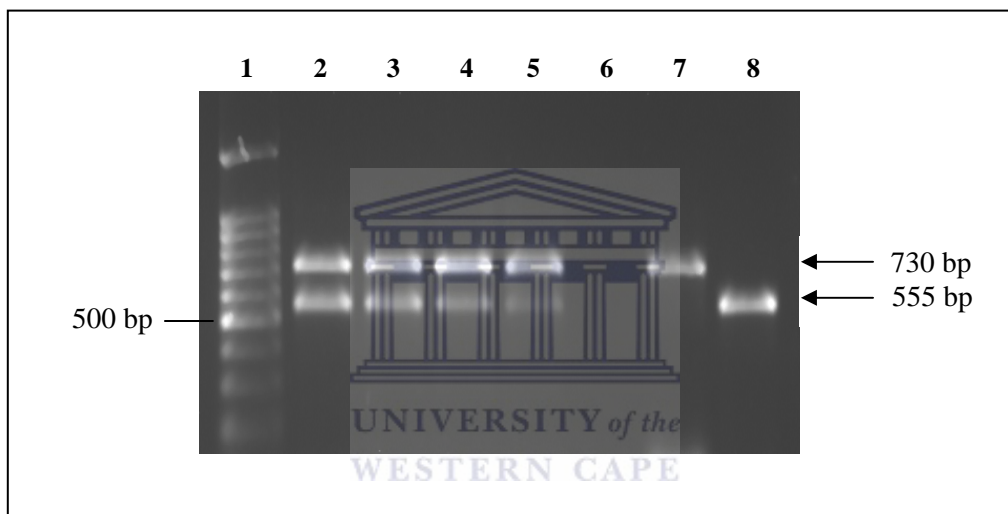


Figure 4.4 The results obtained from multiplex PCR with primer sets *iacF*, *iacR*, *LmonoF* and *LmonoR* in order to determine the optimal concentration of pUC19 for its co-amplification with *L. monocytogenes*. A pure culture of *L. monocytogenes* ScottA was at a constant concentration of 379 ng μl^{-1} with varying concentrations of pUC19 DNA. **Lane 1:** 100 bp DNA ladder (Promega); **lane 2-5:** pUC19 at varying concentrations; **lane 2:** 0.004 pg μl^{-1} ; **lane 3:** 0.003 pg μl^{-1} ; **lane 4:** 0.002 pg μl^{-1} ; **lane 5:** 0.001 pg μl^{-1} ; **lane 6:** negative control (water); **lane 7:** *L. monocytogenes* DNA in the absence of the pUC19 IAC control **lane 8:** pUC19 DNA in the absence of *L. monocytogenes*.

The concentration of the IAC was critical as too high a concentration of IAC DNA template would restrain the amplification of *L. monocytogenes* resulting in a false negative PCR result. However, when the concentration of the IAC was optimal, assay sensitivity was not compromised. When evaluating the inhibitory effect of a range of pUC19 DNA concentrations (0.001-1 pg μl^{-1}) on the amplification of a *L. monocytogenes* pure culture, it was found that at 1 pg μl^{-1} the same assay sensitivity for the co-amplification of the two DNA templates was not attained compared to when a concentration of 0.001 pg μl^{-1} was used (data not shown). At a concentration of 1 pg μl^{-1} , pUC19 was more inhibitory to the amplification of *L. monocytogenes*. Amplification was feasible in the undiluted DNA extract of *L. monocytogenes*; however when performing a dilution series, the lower concentrations of *L. monocytogenes* went undetected. A pUC19 concentration of 0.001 pg μl^{-1} had no inhibitory effect on the amplification of *L. monocytogenes*.

A 0.001 pg μl^{-1} concentration of pUC19 was then co-amplified with a pure culture of *L. monocytogenes* ScottA that was diluted in order to determine the lowest concentration of *L. monocytogenes* that could be detected by the PCR reaction. The detection limit for the presence of *L. monocytogenes* in the pure culture was 0.1 ng μl^{-1} (Figure 4.5).

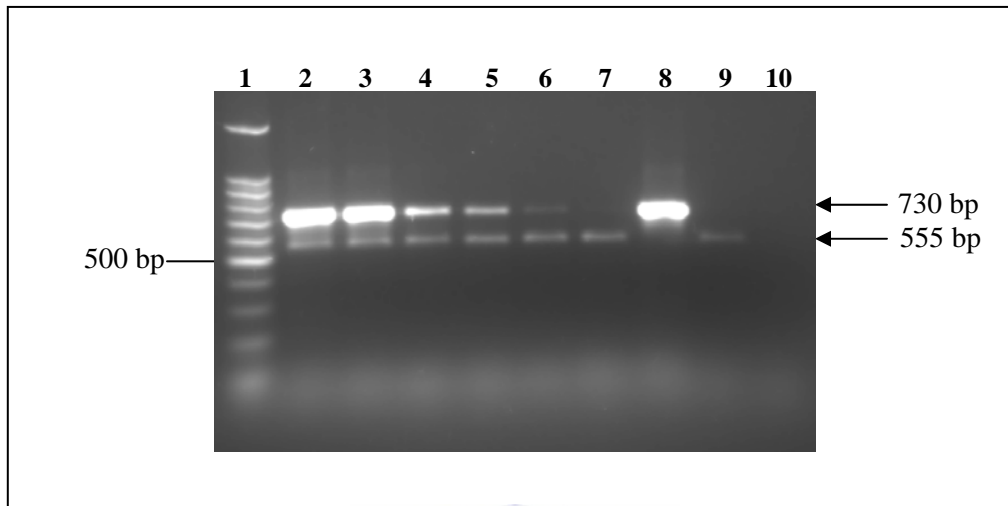


Figure 4.5 Co-amplification of a pure culture of *L. monocytogenes* ScottA and pUC19 DNA using multiplex PCR. A pure culture of *L. monocytogenes* DNA was serially diluted 10-fold with a defined concentration of pUC19 at $0.001 \text{ pg } \mu\text{l}^{-1}$ in every reaction. **Lane 1:** 100 bp DNA ladder (Promega), **lane 2-7:** varying concentrations of *L. monocytogenes*; **lane 2:** $302 \text{ ng } \mu\text{l}^{-1}$; **lane 3:** $76 \text{ ng } \mu\text{l}^{-1}$; **lane 4:** $2.1 \text{ ng } \mu\text{l}^{-1}$; **lane 5:** $1.1 \text{ ng } \mu\text{l}^{-1}$; **lane 6:** $0.3 \text{ ng } \mu\text{l}^{-1}$; **lane 7:** $0.1 \text{ ng } \mu\text{l}^{-1}$; **lane 8:** *L. monocytogenes* DNA in the absence of the pUC19 IAC control; **lane 9:** pUC19 DNA in the absence of *L. monocytogenes*; **lane 10:** negative control (water).

A camembert cheese sample was artificially inoculated with *L. monocytogenes* ScottA, serially diluted (10^0 - 10^{-4}) and co-amplified with the pUC19 IAC. The concentrations of the diluted DNA were acquired using the NanoDrop^R ND-100 spectrophotometer (NanoDrop Technologies). A concentration of $0.3 \text{ ng } \mu\text{l}^{-1}$ *L. monocytogenes* in the cheese sample was detectable by the PCR reaction (Figure 4.6). Since the cheese sample was only diluted to 10^{-3} , no conclusion can be made with regards to the detection limit – a concentration lower than $0.3 \text{ ng } \mu\text{l}^{-1}$ may have been detected, had an extra dilution been

performed. More importantly, these results confirm that a concentration as low as $0.3 \text{ ng } \mu\text{l}^{-1}$ *L. monocytogenes* was detected in a cheese sample by the PCR reaction to which the pUC19 IAC was added.

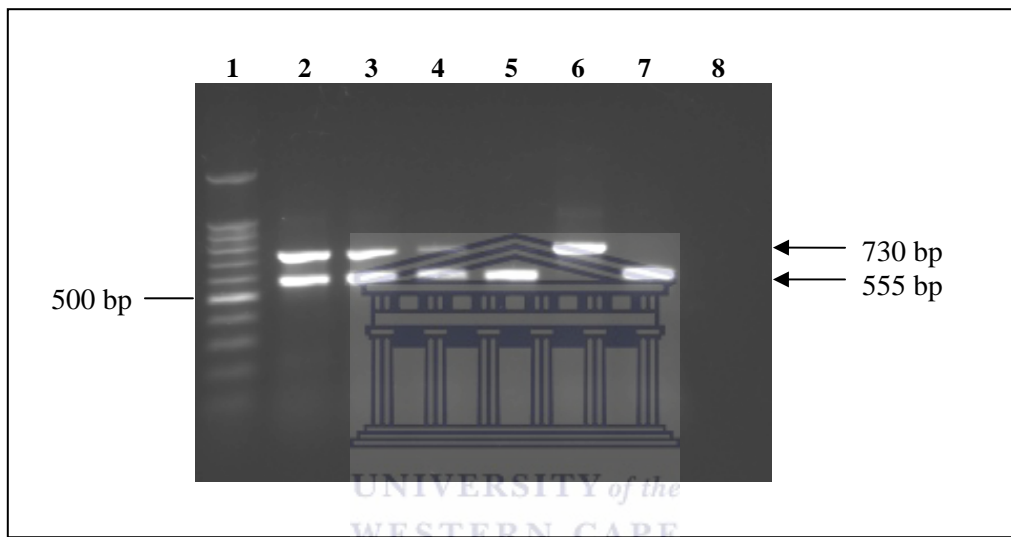


Figure 4.6 A camembert cheese samples spiked with *L. monocytogenes* Scott A. The DNA was diluted 10-fold (10^0 - 10^{-3}) with a constant concentration of pUC19 at $0.001 \text{ pg } \mu\text{l}^{-1}$ in every reaction. **Lane 1:** 100 bp DNA ladder (Promega); **lanes 2-5:** a camembert cheese sample was spiked with *L. monocytogenes* ScottA and serially diluted in $\frac{1}{2}$ strength Fraser broth; **lane 2:** $203 \text{ ng } \mu\text{l}^{-1}$; **lane 3:** $46.9 \text{ ng } \mu\text{l}^{-1}$; **lane 4:** $1.4 \text{ ng } \mu\text{l}^{-1}$; **lane 5:** $0.3 \text{ ng } \mu\text{l}^{-1}$; **lane 6:** *L. monocytogenes* DNA amplified in the absence of the pUC19 IAC; **lane 7:** pUC19 DNA amplified in the absence of *L. monocytogenes*; **lane 8:** negative control (water).

To determine the detection limit of *L. monocytogenes* in spiked food products, a serial dilution of the ostrich food sample was performed and subjected to the PCR reaction without the inclusion of the pUC19 IAC (Figure 4.7). The minimum number of organisms detected by the PCR reaction was 8 cfu ml⁻¹. The sensitivity of the PCR assay was then analyzed by calculating the detection limit for *L. monocytogenes* in the presence of the IAC (Figure 4.8). The target DNA was diluted to determine the lowest number of *L. monocytogenes* that could be amplified in the presence of an IAC. Remarkably, the same detection limit of 8 cfu ml⁻¹ was attained when the pUC19 IAC was included in the PCR reaction. All PCR reactions were repeated several times to confirm results. pUC19, at a concentration of 0.001 pg µl⁻¹, was able to co-amplify with the target DNA of *L. monocytogenes* without compromising the detection limit.

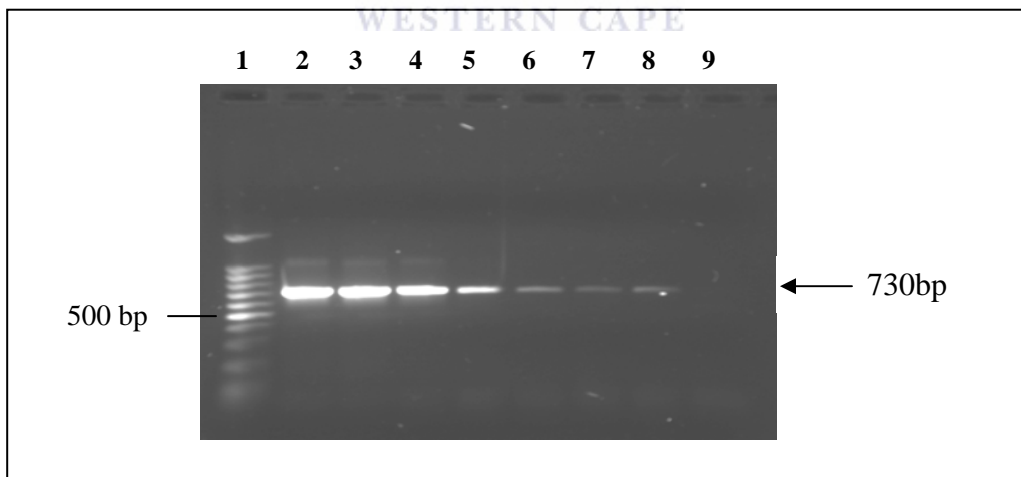


Figure 4.7 Assessment of the detection limit of *L. monocytogenes* in the absence of the pUC19 IAC. An ostrich meat sample was spiked with *L. monocytogenes* ScottA and serially diluted in ½ strength Fraser broth. **Lane 1:** 100 bp DNA ladder (Promega); **lane 2-8:** *L. monocytogenes* serially diluted 10-fold (10^0 - 10^{-6}); **lane 2:** 8×10^6 cfu ml⁻¹; **lane 3:** 8×10^5 cfu ml⁻¹; **lane 4:** 8×10^4 cfu

ml⁻¹; **lane 5**: 8x10³ cfu ml⁻¹; **lane 6**: 8x10² cfu ml⁻¹; **lane 7**: 8x10¹ cfu ml⁻¹; **lane 8**: 8 cfu ml⁻¹; **lane 9**: negative control (water).

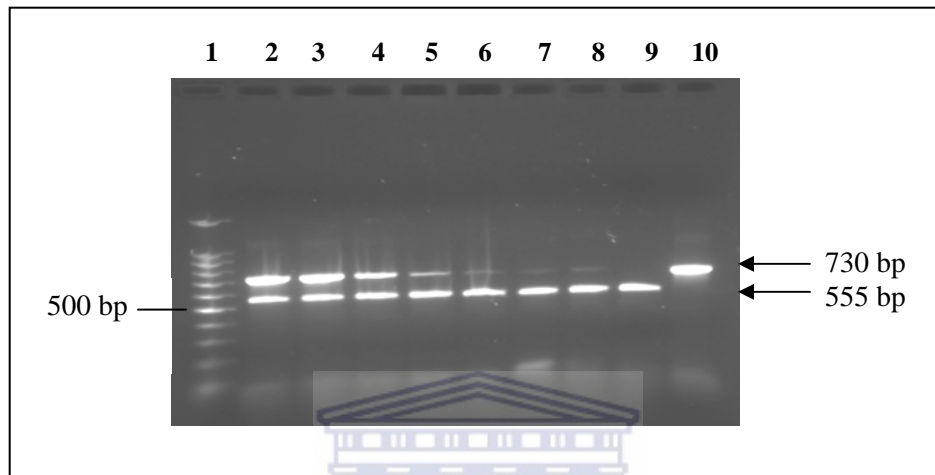


Figure 4.8 The assessment of the inhibitory effect of pUC19 (a constant concentration of 0.001 pg μl^{-1}) on the detection limit of *L. monocytogenes*, determined in figure 4.7. **Lane 1**: 100 bp DNA ladder (Promega); **lane 2-8**: *L. monocytogenes* extracted from spiked ostrich meat that was serially diluted (10^0 - 10^{-6}) and co-amplified with 0.001 pg μl^{-1} pUC19 in each reaction **lane 2**: 8x10⁶ cfu ml⁻¹; **lane 3**: 8x10⁵ cfu ml⁻¹; **lane 4**: 8x10⁴ cfu ml⁻¹; **lane 5**: 8x10³ cfu ml⁻¹; **lane 6**: 8x10² cfu ml⁻¹; **lane 7**: 8x10¹ cfu ml⁻¹; **lane 8**: 8 cfu ml⁻¹; **lane 9**: pUC19 DNA amplified in the absence of *L. monocytogenes*; **lane 10**: *L. monocytogenes* DNA amplified in the absence of the pUC19 IAC.

When the concentration of pUC19 was increased 10-fold to 0.01 pg μl^{-1} , the detection limit for *L. monocytogenes* diluted in 1/2 strength Fraser broth remained 8 cfu ml⁻¹; however the lower concentrations of *L. monocytogenes* were less pronounced on agarose gels compared to when 0.001 pg μl^{-1} of pUC19 was used; proving that the higher the concentration of the IAC, the more inhibitory was its presence to the target DNA

rendering a PCR product with fewer copies of *L. monocytogenes* (Figure 4.9). If used at a higher concentration, the IAC may not detect weak inhibition that would cause false-negative PCR results at extremely low target levels of *L. monocytogenes*. A minimal concentration of IAC DNA was used to prevent competition with *L. monocytogenes*.

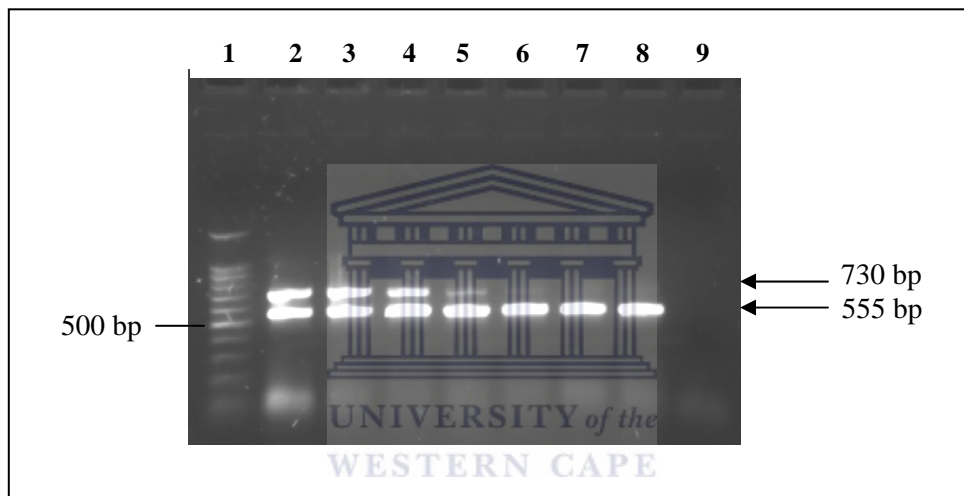


Figure 4.9 Evaluation of the inhibitory effect of a higher concentration of pUC19 on the detection limit of *L. monocytogenes* isolated from spiked ostrich meat. **Lane 1:** 100 bp DNA ladder (Promega); **lane 2-8:** The DNA of *L. monocytogenes* was serially diluted (10^0 - 10^{-6}) in $\frac{1}{2}$ strength Fraser broth and co-amplified with pUC19 at a constant concentration of $0.01 \text{ pg } \mu\text{l}^{-1}$ (10-fold increase); **lane 2:** $8 \times 10^6 \text{ cfu ml}^{-1}$; **lane 3:** $8 \times 10^5 \text{ cfu ml}^{-1}$; **lane 4:** $8 \times 10^4 \text{ cfu ml}^{-1}$; **lane 5:** $8 \times 10^3 \text{ cfu ml}^{-1}$; **lane 6:** $8 \times 10^2 \text{ cfu ml}^{-1}$; **lane 7:** $8 \times 10^1 \text{ cfu ml}^{-1}$; **lane 8:** 8 cfu ml^{-1} ; **lane 9:** negative control (water)

The results of this study have demonstrated that incorporating an IAC into the PCR reaction has not compromised the specificity or sensitivity of the assay. In retrospect, the sensitivity and reliability of the PCR reaction increased as samples identified or presumed

to be negative could be retested with an IAC to confirm whether PCR failure occurred or whether it was a real negative PCR result. Once the optimal concentration of the IAC was determined, the IAC was used for monitoring PCR-inhibiting components present in food samples that may interfere with the detection of food-borne pathogens. The results obtained in this study proved that the IAC constructed was suitable for its co-amplification with *L. monocytogenes* without loss of the detection limit.



4.5 CONCLUSION

For PCR results to be interpreted correctly, it is important to know whether PCR failure occurred or whether it was a real negative PCR result. Inhibitory substances in food products may influence the outcome of the PCR reaction by lowering or completely preventing amplification. The consequence of a false-negative result for the presence of *Listeria monocytogenes* in food products is severe.

The use of an IAC provides essential information about the presence of inhibitory factors in food products and allows for the interpretation of a negative result. The method of IAC construction described in this study is simple, easy and universal. The aim of this study, to develop an IAC that would co-amplify with the *hly* gene of *L. monocytogenes* in order to eliminate the occurrence of false-negative PCR results, was achieved.

There were a few important procedures that had to be followed when designing the IAC. It had to be confirmed that the IAC was amplified and detected and thereafter that the simultaneous amplification of the pUC19 IAC and target *L. monocytogenes* sequence occurred. The concentration of the IAC had to be optimized to prevent inhibition of the target DNA. The detection limit of the IAC had to be verified in order to determine its optimal concentration for use in the PCR reaction and furthermore that the IAC would function in the sample matrix chosen had to be authenticated. With reference to the above, pUC19 was found to co-amplify with *L. monocytogenes* at an optimized

concentration of $0.001 \text{ pg } \mu\text{l}^{-1}$ without compromising the sensitivity or specificity of the PCR reaction. As few as 8 cfu ml^{-1} *L. monocytogenes*, extracted from spiked ostrich meat, could be detected by the PCR reaction when no pUC19 IAC was added; taking into consideration that the same detection limit was attained when the pUC19 IAC was co-amplified with *L. monocytogenes*. It was imperative to optimize the concentration of the pUC19 IAC, since assay sensitivity was compromised when pUC19 was used at a concentration of $1 \text{ pg } \mu\text{l}^{-1}$.

When designing the IAC, it was imperative to choose a sequence that differed in size compared to the target DNA, so that electrophoresis on agarose gel could provide a good distinction between the two products. A small difference in fragment length can complicate the objective assessment of the results and could possibly lead to a false-negative or false positive-result.

The pUC19 IAC that was constructed provided the assurance that negative test results were truly negative. The use of the optimal pUC19 IAC concentration increased the reliability of the PCR reaction and has proved to be useful for food diagnostics. It can be concluded that the IAC-PCR test designed in this study could form the basis of an accurate, standardized and robust screening method for the presence of *L. monocytogenes* in food products.

CHAPTER 5

Differentiation of the *Listeria* genus in food products by the denaturing gradient gel electrophoresis method

5.1 ABSTRACT

Aim: The aim of this study was to differentiate between different species of the genus *Listeria* within food products, namely *Listeria monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seelgeri*, *L. ivanovii* and *L. grayi* as well as *L. monocytogenes* serotypes NCTC 11944, ScottA (UWC L1), NCTC 7973 and NCTC 4855, by using polymerase chain reaction (PCR) and PCR-based denaturing gradient gel electrophoresis (DGGE). The ability of DGGE to adequately differentiate between *Listeria* spp. and *L. monocytogenes* serotypes was investigated.

Materials and methods: The PCR-based DGGE method was used to distinguish *Listeria monocytogenes* from *L. innocua*, *L. welshimeri*, *L. seelgeri*, *L. ivanovii* and *L. grayi*. These reference strains were subjected to conventional testing and identification methods such as selective plating on Oxford agar and API-*Listeria* analysis. The *L. monocytogenes* DNA that was extracted from spiked ostrich meat and camembert cheese samples together with pure cultures of *L. innocua*, *L. welshimeri*, *L. seelgeri*, *L. ivanovii* and *L.*

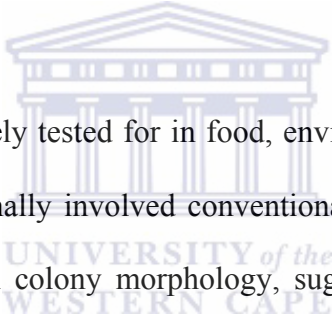
grayi were analyzed using PCR-based DGGE, to obtain sample specific fingerprints. The fingerprints provided a pattern of bands corresponding to a specific *Listeria* spp. or *L. monocytogenes* serotype occurring in the analyzed food sample or pure culture.

Conclusion: The PCR-based DGGE technique has proved to be reliable and effectual for the differentiation of *Listeria* spp. and *L. monocytogenes* serotypes extracted from food products; providing better distinction between *L. monocytogenes* and *L. innocua* compared to the result obtained by electrophoresis on an agarose gel.

Significance and impact of the study: Adequate differentiation between *Listeria* spp. was observed with DGGE analysis. The protocol for PCR-DGGE analysis of food samples in this study has proved to be reproducible and reliable for food diagnostic purposes, especially since many conventional methods provided presumptive identification. The application of the PCR-DGGE method allowed for reliable monitoring of *Listeria* spp. and serotypes in food products and demonstrated the great potential that this method had over other conventional and molecular techniques.

5.2 INTRODUCTION

The sub-division of the genus *Listeria* into serotypes has been useful for practical and epidemiological purposes, since members of the genus *Listeria* have been implicated in listeriosis outbreaks (Cocolin *et al.* 2002). Many subtyping methods have proven useful in differentiating *Listeria monocytogenes* below its species level. Serotyping has divided *L. monocytogenes* into 13 serotypes based on its somatic and flagellar antigens (Nadon *et al.* 2001).



Listeria monocytogenes is widely tested for in food, environmental and clinical samples whereby identification traditionally involved conventional culture methods followed by species identification based on colony morphology, sugar fermentation and hemolytic properties (Gasnov *et al.* 2005). The limitations when using these biochemical standard methods for species identification is that the differentiation between species is not always achieved along with the fact that the process is time consuming and laborious (Cocolin *et al.* 2002). Many diagnostic tests have been developed to differentiate *L. monocytogenes* from the other species of the genus. Although the other serotypes are also implicated in the contamination of food, only serotypes 1/2a, 1/2b and 4b belonging to *L. monocytogenes* are responsible for 90% of listeriosis outbreaks (Gasnov *et al.* 2005).

Denaturing gradient gel electrophoresis has been very useful for epidemiological studies and is able to generate a profile showing the genetic diversity of a microbial population in

a specific environment (Ercolini 2004). The DGGE technique has shown to be very powerful in differentiating mutational variations between DNA fragments, since the variant will melt at a specific location along the gradient (Van Orsouw *et al.* 1998; Hayes *et al.* 1999; Fujimoto *et al.* 2003). The accuracy of the DGGE technique greatly depends on the design of the PCR primers (including the length, position and nucleotide sequence of the GC-clamp) and the melting domain of the DNA fragments (Van Orsouw *et al.* 1998; Hayes *et al.* 1999).

In a study undertaken by Cocolin *et al.* (2004), the microbial profile of fresh sausage stored at 4°C from the product day to day 10 of storage could be evaluated by PCR-DGGE. This technique allowed for the changes in bacterial and yeast population to be studied in detail over the 10 day storage. To determine the effects of refrigeration (4°C) on the changes in bacterial populations in raw milk samples, the DGGE approach was also employed (Lafarge *et al.* 2004).

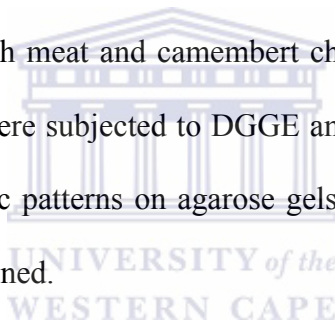
The principle of the DGGE technique is as follows. The two strands of the DNA molecule melt or separate when heat or a chemical denaturant is applied. The temperature at which the double strand melts is influenced by two factors namely, GC rich domains which melt at a higher temperature compared to AT rich domains which denatures more easily; and secondly, the attraction between neighboring bases of the same strand namely, stacking interactions. The degree of stacking is determined by the order of bases on the strand. Therefore, depending on the nucleotide sequence, the molecules which differ by

only one nucleotide will have several melting domains. A single base change may affect the stacking interaction enough to alter the melting temperature (T_m) by over 1°C (Hope 2004).

The DGGE technique uses a special form of acrylamide gels that can separate small (200-700 bp) genomic fragments of the same or similar length but with different base composition. The gel is poured in a gradient increasing in denaturing strength, provided by formamide and urea, in the direction of the electrophoretic run (100% denaturing strength consists of 40% formamide and 7M urea) (Hayes *et al.* 1999; Ercolini 2004). The melting behaviour of a DNA fragment determines its migration pattern in the gel. The mobility of the DNA molecule changes at the concentration at which the DNA strands with a low melting domain separate resulting in a partially single stranded molecule (Hope 2004). A partially denatured fragment moves much more slowly or becomes entangled in the gel matrix resulting in no movement through the polyacrylamide gel compared to a single- or double-stranded fragment. When DNA is loaded into the denaturing gel, the double-stranded DNA molecules become partially melted and their mobility decreases within the gel. However, if the double-stranded DNA molecules become completely melted into single strands, their mobility increases. A good resolution results when the DNA molecules do not completely separate (Ercolini 2004). To prevent total denaturation of the DNA molecule a GC rich sequence, a GC-clamp with a high melting domain, is attached to one primer before PCR amplification (Hayes *et al.* 1999; Chang Bioscience 2004).

The PCR reaction was used to amplify the *iap* gene which encodes the invasion associated protein (p60) common in the *Listeria* spp. included in this study, by using primers specific for this region. The *iap* gene was demonstrated to be a reliable PCR target for the differentiation of *Listeria* spp. It has conserved regions at the 5' and 3' ends and a species-specific internal region (Bubert *et al.* 1999).

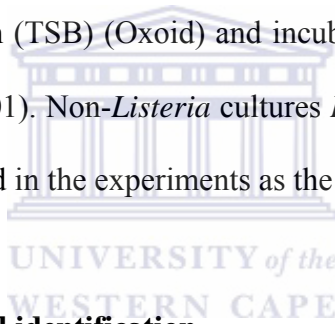
The aim of this study was to test the usefulness of the PCR-DGGE technique as a rapid and effective method for the screening of *Listeria* spp. and *L. monocytogenes* serotypes in pure cultures and spiked ostrich meat and camembert cheese samples. PCR products of the *iap* gene of *Listeria* spp. were subjected to DGGE analysis and the results were then compared to the electrophoretic patterns on agarose gels in order to draw a comparison between the identification obtained.



5.3 MATERIALS AND METHODS

5.3.1 Reference strains

Glycerol stocks for the *Listeria* species, *Listeria monocytogenes* NCTC 11944, ScottA (UWC L1), NCTC 7973 and NCTC 4855, *L. innocua*, *L. ivanovii*, *L. welshimeri*, *L. grayi* and *L. seeligeri* were used in this study. To resuscitate the cells, 100 µl was inoculated into 10 ml Tryptone Soy Broth (TSB) (Oxoid) and incubated at 37°C overnight (Norton and Batt 1999; Smith *et al.* 2001). Non-*Listeria* cultures *Escherichia coli* and *Salmonella enteritidis* were grown and used in the experiments as the negative controls.



5.3.2 Traditional isolation and identification

5.3.2.1 API-Listeria

Before the reference strains of the *Listeria* genus, *Listeria innocua*, *L. ivanovii*, *L. welshimeri*, *L. grayi* and *L. seeligeri*, were used for DGGE analysis, the API-Listeria (BioMérieux) test was performed, according to the manufacturer's instructions, to ensure that these reference strains were indeed that which were specified. These reference strains were streaked onto Oxford agar (Oxoid), incubated at 37°C overnight and *Listeria* positive colonies were then streaked onto Tryptone Soy Agar (TSA) (Oxoid) and

incubated overnight at 37°C (Gouws and Liedemann 2005). Presumptive positive colonies from the TSA plate were resuspended in suspension buffer (provided with API-*Listeria* kit). This suspension was distributed into the microtubes, 50 µl for all the tests and 100 µl for the ‘DIM’ test, which was required to differentiate *L. monocytogenes* from *L. innocua*. The base of the tray was overlaid with sterile distilled water to prevent drying out of the strip. Following incubation at 37°C for 18 h, the results were interpreted using the API-*Listeria* manual. A drop of ZYM B reagent (provided with API-*Listeria* kit) was added to the ‘DIM’ test to differentiate *L. monocytogenes* from *L. innocua* based on a colour reaction (Billie *et al.* 1992).

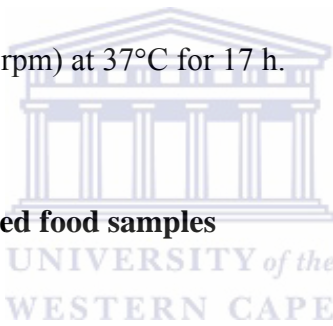


5.3.2.2 Gram Stain

To determine the Gram reaction and morphology of the reference strains, a Gram stain was performed. A colony from the TSA plate (as prepared in section 5.3.2.1) was smeared onto a slide using a sterilized loop together with a drop of sterile distilled water and air-dried. The bacterial smear was then heat fixed and treated with crystal violet for 1 min, iodine for 1 min, ethanol for 15 s and lastly safranin for 1 min. The slide was rinsed with water between each treatment. The slide was then air-dried and viewed under a light microscope (Johnson 2003).

5.3.3 Sample preparation of spiked food products

The food samples (25 g), ostrich meat and camembert cheese, were prepared using sterilized instruments and spiked with *L. monocytogenes* serotypes NCTC 11944, ScottA, NCTC 7973 and NCTC 4855 and then homogenized in 225 ml of Buffered Peptone Water (BPW) (Oxoid) (Löfström *et al.* 2004) for 60 s using a Stomacher 400 laboratory blender (Seward Ltd). Samples were incubated at 37°C for 5 h. Thereafter, 0.1 ml was extracted and inoculated into 10 ml ½ strength Fraser broth (Oxoid). This suspension was put in a shaking incubator (114 rpm) at 37°C for 17 h.

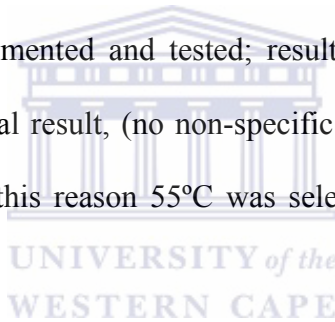


5.3.4 DNA isolation from spiked food samples

A method modified by Agarsborg *et al.* (1997) was used for DNA isolation. A 2.0 ml aliquot culture was transferred to a 2.0 ml eppendorf tube. Cultures were centrifuged at 9000 x g for 10 min. The pellet was resuspended in 400 µl sterile distilled water to which 400 µl 2% Triton-X-100 (BDH Chemicals Ltd) was added and the contents mixed for 5 s. This suspension was left at room temperature for 10 min, thereafter incubated at 100°C for 10 min and then centrifuged at 9000 x g for 4 min. The supernatant was transferred to a sterile eppendorf tube and 2 µl of this crude cell lysate was used for PCR amplification.

5.3.5 Gradient PCR

To optimize the annealing temperature for the cycling parameters, gradient PCR using the Eppendorf Mastercycler gradient (Merck) was used. Initially the cycling parameters presented by Cocolin *et al.* (2002) was implemented; however, faint non-specific bands were amplified resulting in the gradient PCR technique being employed to find an annealing temperature that was more optimal for primer annealing to the target DNA without the amplification of non-specific DNA fragments. A temperature range of 40°-50°C and 50°-60°C was implemented and tested; resulting in no non-specific binding from 50°C upwards. An optimal result, (no non-specific amplification) was observed at 55°C (result not shown). For this reason 55°C was selected as the optimum annealing temperature.



5.3.6 PCR amplification of the *iap* gene

The PCR reaction was used to amplify the *iap* gene which encodes the invasion associated protein, p60, by using primers specific for this region. The primers that were used for PCR-DGGE analysis have a GC-clamp attached to the 5' end of the forward primer. These primers amplify a 457 bp region in *L. monocytogenes* and 472 bp region in *L. innocua* while the sizes of the amplicons for the other 3 species are 601 bp for *L. seeligeri* and 610 bp for both *L. ivanovii* and *L. welshimeri*. Distinction of the PCR products was made possible by electrophoresis on agarose gels. The sequence of the List

U1GC primer and List U2 primer used (Cocolin *et al.* 2002) are listed in Table 5.1. Careful optimization of constituent quantities as well as thermal cycling parameters took place. For a 50 µl reaction, the mixture contained: 1 X PCR buffer (Celtic Molecular Diagnostics, Bioline), MgCl₂ (final concentration 5mM) (Celtic Molecular Diagnostics, Bioline), dNTP's (final concentration 200 µM) (Roche Diagnostic), List U1GC and List U2 primers (final concentration 0.3 µM each) (Whitehead Scientific, IDT), 1U *Biotaq* DNA polymerase (Celtic Molecular Diagnostics, Bioline) and 2 µl template DNA (10⁰). Amplification was carried out in a thermal cycler GeneAmp[®] PCR system 2700 (Applied Biosystems) with the following optimized programme: Initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 40 s and extension at 72°C for 1 min with a final extension step at 72°C for 5 min. These cycling parameters were optimized using gradient PCR. The cycling parameters described by Cocolin *et al.* (2002), which were used initially, was as follows: Initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 36°C for 2 min, extension at 72°C for 3 min with a final extension step at 72°C for 7 min.

The PCR products (8 µl) were subjected to electrophoresis on a 0.8% agarose D-1 LE gel (Whitehead Scientific) prior to DGGE analysis and were visualized by staining with Ethidium Bromide staining. The amplified PCR products were viewed using the Alphaimager[®] HP system (AlphaInnotech Corporation). Gel pictures were acquired using the AlphaEase FC[™] software version 4.0.0.

5.3.7 Species-specific PCR

In order to confirm whether the *L. monocytogenes* serotypes NCTC 11944, ScottA, NCTC 7973 and NCTC 4855 were indeed *L. monocytogenes* strains, they were subjected to PCR analysis whereby characterization was based on whether amplification of the *hly* gene (a virulence gene specific for *L. monocytogenes*) had occurred. For a 25 µl reaction, the mixture contained: 1 X PCR buffer (Celtic Molecular Diagnostics, Bioline), MgCl₂ (final concentration 5mM) (Celtic Molecular Diagnostics, Bioline), dNTP's (final concentration 200 µM) (Roche Diagnostic), LmonoF and LmonoR primers (final concentration 0.3 µM each) (Whitehead Scientific, IDT), 1U *Biotaq* DNA polymerase (Celtic Molecular Diagnostics, Bioline) and 1 µl template DNA (10⁰). The sequence for LmonoF and LmonoR is illustrated in Table 5.1. These primers amplify a 730 bp region of the *hly* gene (Blaise and Phillippe 1995).

Amplification was carried out in a thermal cycler GeneAmp[®] PCR system 2700 (Applied Biosystems) with the following optimized programme: Initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 40 s and extension at 72°C for 1 min with a final extension step at 72°C for 2 min. The PCR products (7 µl) underwent electrophoresis on a 1% agarose D-1 LE gel (Whitehead Scientific) and was visualized by staining with Ethidium Bromide. The amplified PCR products were viewed using the AlphaImager[®] HP system (AlphaInnotech Corporation). Gel pictures were acquired using the AlphaEase FC[™] software version 4.0.0.

Table 5.1 Primer sets used for PCR-DGGE analysis

	Primer sequence
List U1GC Forward primer	5' – GCC AGC GGC CCG GCG CGG GCC CGG CGG CGG GGG CCG CGG C ATG TCA TGG AAT AA – 3'
List U2 Reverse primer	5' – GCT TTT CCA AGG TGT TTT T – 3'
LmonoF Forward primer	5' – CAT TAG TGG AAA GAT GGA ATG – 3'
LmonoR Reverse primer	5' – GTA TCC TCC AGA GTG ATC GA – 3'

GC-clamp is identified in bold



5.3.8 Denaturing gradient gel electrophoresis

DGGE analysis was performed using the BioRad DCode™ Universal Mutation Detection System (Biorad Laboratories, USA). The following solutions were made for DGGE analysis. A 100 ml 0% denaturing gel solution comprised 40% (v/v) bis-acrylamide (37:5:1), 2% (v/v) 50 X TAE (Tris Acetic acid EDTA) buffer and 78% (v/v) sterile distilled water. This solution was filtered through a 0.45µ filter and stored at 4°C. A 100 ml 100% denaturing gel solution comprised 40% (v/v) bis-acrylamide (37:5:1), 2% (v/v) 50 X TAE buffer, 40% (v/v) deionized formamide (Sigma) and 7.0 M urea (Qiagen). The solution was placed in a waterbath prior to use to dissolve all the ingredients, but stored at 4°C.

A 0-45% linear denaturant gradient gel was prepared using a low and a high solution. The low solution comprised 3.25 ml 100% solution, 9.75 ml 0% solution, 24 μ l N,N,N',N'-tetramethyl-ethylenediamine (TEMED) and 236 μ l 10% (w/v) ammonium persulphate (APS) and the high solution comprised 5.85 ml 100% solution, 7.15 ml 0% solution, 24 μ l TEMED and 236 μ l 10% (w/v) APS. A stacking solution was also prepared using 5 ml of 0% denaturing solution, 9 μ l TEMED and 91 μ l 10% APS. After the gel was poured it was left to solidify for approximately 1 h. The buffer tank was set at 60°C and once this temperature was acquired, 20 μ l PCR product together with 8 μ l 6 X loading buffer (100 ml volume comprised 0.05% (w/v) bromophenol blue, 40% (w/v) sucrose, 0.5 M EDTA (pH 8) and 0.5% (w/v) SDS) was loaded into the wells. The voltage was turned on to 130 V and electrophoresis took place for 5 h. Thereafter, the gel was stained in a 313 ml 1 X TAE buffer and 32 μ l ethidium bromide (EtBr) solution for 30 min. The DGGE profile was viewed using the Alphaimager[®] HP system (AlphaInnotech Corporation) and the pictures were acquired using the AlphaEase FC[™] software version 4.0.0.

5.4 RESULTS AND DISCUSSION

In this study PCR-DGGE analysis was employed to differentiate between the *Listeria* genus and *L. monocytogenes* serotypes isolated from pure cultures and food samples.

When conventional detection methods were used in this study, no distinction between the *Listeria* spp. was obtained. When the reference strains, *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. grayi*, were streaked onto Oxford agar, it created the presumption that all strains belonged to the genus *Listeria* since esculin hydrolysis was evident due to the production of grey-green colonies with a black halo. This is a characteristic feature for the presence of any *Listeria* spp. however; some other organisms are able to utilize esculin, namely, *Bacillus* spp. and *Enterococcus*. They mimic the appearance of *Listeria* spp. therefore further tests may need to be performed in order to confirm presumptive results (Gasanov *et al.* 2005).

The API-*Listeria* test was used to further confirm presumptive results, which allowed for the distinction between species of the genus *Listeria*. The reference strains, *L. monocytogenes*, *L. innocua*, *L. grayi*, *L. welshimeri* and *L. seeligeri* that were subjected to the API-*Listeria* test produced the following results. The reference strain for *L. monocytogenes* was identified as *L. monocytogenes*, *L. innocua* as *L. innocua*, *L. grayi* as *L. grayi*, *L. welshimeri* as *L. welshimeri*, however; the reference strains presumed to be *L. ivanovii* and *L. seeligeri* were identified as *L. monocytogenes* (Table 5.2). When these

strains were used in DGGE analysis, their migration pattern and profile were exactly that of the reference strain for *L. monocytogenes*, concluding that they were indeed a *L. monocytogenes* strain and not that of *L. ivanovii* or *L. seeligeri* (Figure 5.5). To distinguish *L. monocytogenes* from *L. ivanovii* or *L. seeligeri* is of utmost importance, especially to the food industry, since of the species is *L. monocytogenes* the only type linked with food-borne listeriosis and capable of causing infections like septicemia, meningitis, abortion and gastroenteritis (Rossmann *et al.* 2006).



Table 5.2 A comparison of the reference strains subjected to biochemical and molecular methods.

Reference strain	API analysis	PCR-DGGE analysis
<i>L. welshimeri</i>	<i>L. welshimeri</i>	<i>L. welshimeri</i>
<i>L. grayi</i>	<i>L. grayi</i>	N/A
<i>L. innocua</i>	<i>L. innocua</i>	<i>L. innocua</i>
<i>L. seeligeri</i>	<i>L. monocytogenes</i>	<i>L. monocytogenes</i>
<i>L. ivanovii</i>	<i>L. monocytogenes</i>	<i>L. monocytogenes</i>
<i>L. monocytogenes</i> *NCTC 11944	N/A	<i>L. monocytogenes</i>
<i>L. monocytogenes</i> ScottA	N/A	<i>L. monocytogenes</i>
<i>L. monocytogenes</i> *NCTC 7973	N/A	<i>L. monocytogenes</i>

*** NCTC – National Collection of Type cultures**

Strains identified in bold were identified differently by the API-*Listeria* and PCR-DGGE analysis.

For PCR analysis, the primer pair was designed to amplify the *iap* gene, encoding the invasion-associated protein p60, common in *Listeria* spp. due to its high or complete homology to the *iap* genes of the five species namely, *Listeria monocytogenes*, *L. welshimeri*, *L. innocua*, *L. ivanovii* and *L. seeligeri*. The forward primer was completely homologous to all five species included in this study while the reverse primer was homologous only to *L. monocytogenes*, with a mismatch in only one nucleotide position

for the remaining four species. For this reason amplicons with different sizes were produced on agarose gels based on the fact that regions with differences in sequences were amplified (Cocolin *et al.* 2002). The primers were designed to amplify a 457 bp DNA fragment in *L. monocytogenes*, a 472 bp DNA fragment in *L. innocua*, a 601 bp DNA fragment in *L. seeligeri* and 610 bp DNA fragments in *L. ivanovii* and *L. welshimeri*. The primers used in this study proved to be very reliable for its use in PCR-DGGE analysis since it was very specific for *Listeria* spp. without amplifying DNA of the non-*Listeria* cultures that were included in this study, namely *Salmonella enteritidis* and *Escherichia coli* (Figure 5.1).



Initially the protocol for the PCR cycle parameters by Cocolin *et al.* (2002) was employed whereby an annealing temperature of 36°C was used (Figure 5.1 and 5.2). Clearly many faint non-specific DNA fragments were amplified. To optimize the conditions for primer annealing and reduce non-specific amplification, gradient PCR was employed at a temperature range of 40°-60°C (Figure 5.3). Optimal conditions were created at 55°C and this was the annealing temperature selected for subsequent experiments (Figure 5.4 and 5.5). Where more than one band for a single species is still present after optimization of the PCR cycle parameters, may be indicative of different strains or a heterogeneous rDNA operon being present (Theunissen *et al.* 2005).

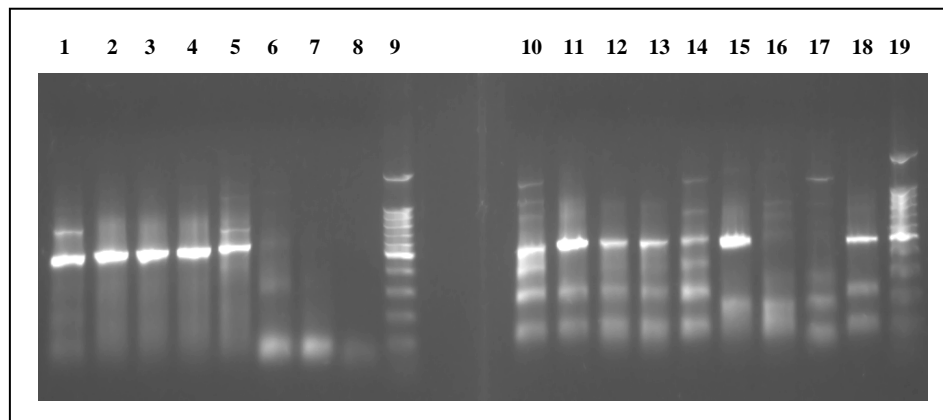


Figure 5.1 Agarose gel electrophoresis of the products obtained after amplification of the *iap* gene from *L. monocytogenes* serotypes and *L. innocua*. PCR products of *Listeria* pure cultures (lane 1-5) and spiked meat and cheese samples (lane 11-15, 18). The protocol by Cocolin *et al.* (2002) was employed using an annealing temperature of 36°C. Amplicon size 457 bp except for lane 1 and 11 (*Listeria innocua*) which was 472 bp. **Lane 1:** *Listeria innocua* (UWC isolate); **lane 2:** *L. monocytogenes* NCTC 11944; **lane 3:** *L. monocytogenes* ScottA; **lane 4:** *L. monocytogenes* NCTC 7973; **lane 5:** *L. monocytogenes* NCTC 4855; **lane 6:** negative control *Escherichia coli*; **lane 7:** negative control *Salmonella enteritidis*; **lane 8:** negative control (water); **lane 9:** 100 bp DNA ladder (Promega); **lane 10:** 100 bp DNA ladder (Promega); **lane 11:** ostrich meat spiked with *L. innocua*; **lane 12:** ostrich meat spiked with *L. monocytogenes* NCTC 11944; **lane 13:** ostrich meat spiked with *L. monocytogenes* ScottA; **lane 14:** ostrich meat spiked with *L. monocytogenes* NCTC 7973; **lane 15:** ostrich meat spiked with *L. monocytogenes* NCTC 4855; **lane 16:** negative control *Escherichia coli*; **lane 17:** negative control *Salmonella enteritidis*; **lane 18:** camembert cheese spiked with *L. monocytogenes* ScottA; **lane 19:** 100 bp DNA ladder (Promega).

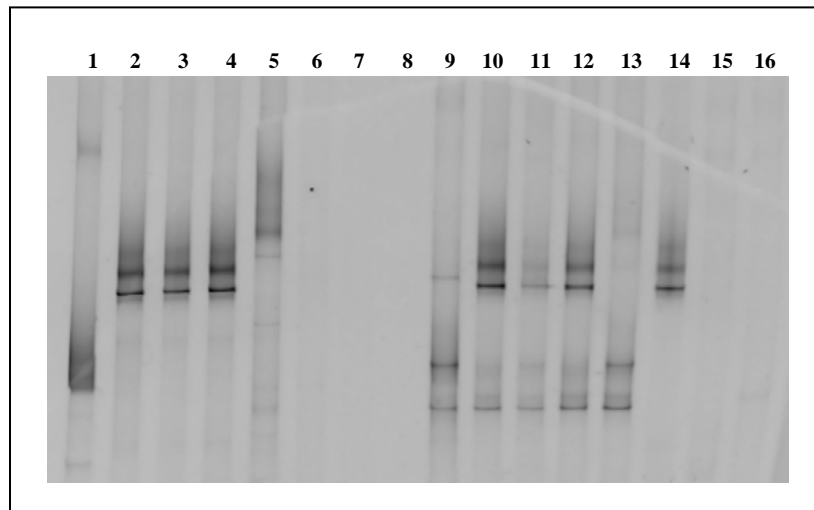


Figure 5.2 DGGE profile of *Listeria* pure cultures (lane 1-5) and spiked food products (lane 9-14) on a polyacrylamide gel. The protocol by Cocolin *et al.* (2002) was employed. **Lane 1:** *Listeria innocua*; **lane 2:** *L. monocytogenes* NCTC 11944; **lane 3:** *L. monocytogenes* ScottA; **lane 4:** *L. monocytogenes* NCTC 7973; **lane 5:** *L. monocytogenes* NCTC 4855; **lane 6:** negative control *Escherichia coli*; **lane 7:** negative control *Salmonella enteritidis*; **lane 8:** negative control (water); **lane 9:** ostrich meat spiked with *L. innocua*; **lane 10:** ostrich meat spiked with *L. monocytogenes* NCTC 11944; **lane 11:** ostrich meat spiked with *L. monocytogenes* ScottA; **lane 12:** ostrich meat spiked with *L. monocytogenes* NCTC 7973; **lane 13:** ostrich meat spiked with *L. monocytogenes* NCTC 4855; **lane 14:** camembert cheese spiked with *L. monocytogenes* ScottA; **lane 15-16:** camembert cheese presumed to be contaminated with *L. monocytogenes*

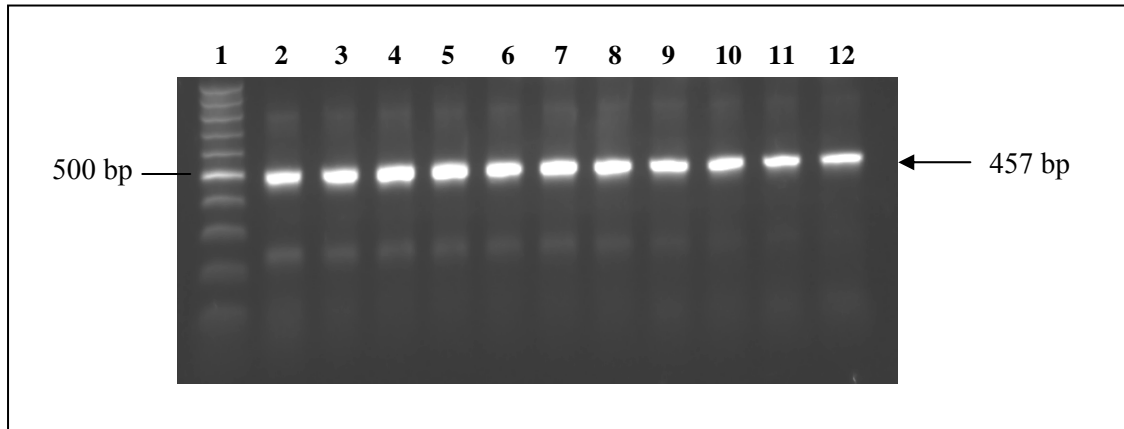


Figure 5.3 Gradient PCR (40°-50°C) of an ostrich meat sample spiked with *L. monocytogenes* NCTC 7973 using the *iap* gene as a target for PCR amplification. **Lane 1:** 100 bp DNA ladder (Promega); **lanes 2-12:** annealing temperatures of 40°-50°C were employed to find the temperature that was more optimal for primer annealing to the target DNA without the amplification of non-specific DNA. **lane 2:** 40.1°C; **lane 3:** 40.7°C; **lane 4:** 41.5°C; **lane 5:** 42.7°C; **lane 6:** 44°C; **lane 7:** 45.3°C; **lane 8:** 46.7°C; **lane 9:** 48°C; **lane 10:** 49.1°C; **lane 11:** 49.9°C; **lane 12:** 50.4°C

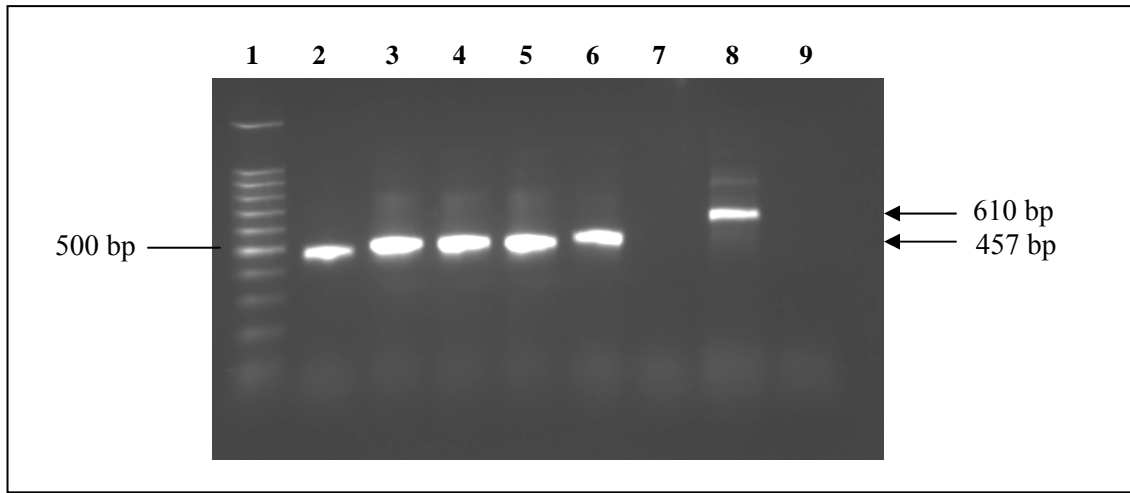


Figure 5.4 Result of the optimized PCR protocol and PCR cycle parameters with an annealing temperature of 55°C. **Lane 1:** 100 bp DNA ladder (Promega); **lane 2-8:** PCR products of pure cultures of *Listeria*; **lane 2:** *Listeria innocua*; **lane 3:** *L. monocytogenes* NCTC 11944; **lane 4:** *L. monocytogenes* ScottA; **lane 5:** *L. monocytogenes* NCTC 7973; **lane 6:** *L. monocytogenes* NCTC 4855; **lane 7:** *L. grayi*; **lane 8:** *L. welshimeri*; **lane 9:** negative control (water).

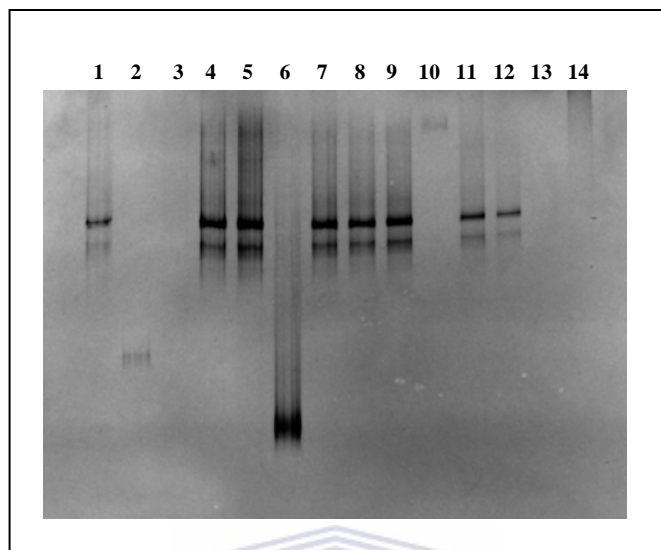


Figure 5.5 DGGE analyses on a polyacrylamide gel of *Listeria* pure cultures and *L. monocytogenes* extracted from spiked foods samples. The optimized PCR protocol and cycle parameters were used in the PCR reaction to reduce non-specific binding and amplification. **Lane 1:** *L. monocytogenes* reference strain; **lane 2:** *L. welshimeri*; **lane 3:** *L. grayi*; **lane 4:** presumptive *L. ivanovii*; **lane 5:** presumptive *L. seeligeri*; **lane 6:** *L. innocua*; **lane 7:** pure culture *L. monocytogenes* NCTC 11944; **lane 8:** pure culture *L. monocytogenes* ScottA; **lane 9:** pure culture *L. monocytogenes* NCTC 7973; **lane 10:** pure culture *L. monocytogenes* NCTC 4855; **lane 11:** ostrich meat spiked with *L. monocytogenes* ScottA; **lane 12:** camembert cheese spiked with *L. monocytogenes* ScottA; **lane 13:** negative control (water); **lane 14:** negative control *Salmonella enteritidis*

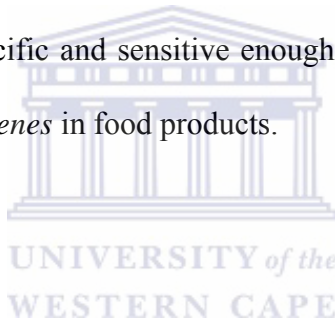
Agarose gel electrophoresis of the PCR products showed no distinction between the *L. monocytogenes* serotypes included in this study. A 457 bp product, characteristic of all *L. monocytogenes* serotypes, was observed. *L. monocytogenes* serotypes NCTC 11944, ScottA and NCTC 7973 all had the same profile and migratory pattern on the DGGE gel; although the *L. monocytogenes* serotype NCTC 4855 had a different denaturing profile

compared to the others. To confirm that this strain was indeed a *L. monocytogenes* serotype, species-specific PCR amplification targeting the *hly* gene, a virulence gene present in *L. monocytogenes*, was employed and amplification of the target 730 bp product resulted (result not shown). This confirmed that *L. monocytogenes* NCTC 4855 was indeed a *L. monocytogenes* serotype; which had a different migratory pattern to the other *L. monocytogenes* serotypes when subjected to DGGE analysis. The fact that *L. monocytogenes* serotypes NCTC 11944, ScottA and NCTC 7973 all had the same profile and migratory pattern on the DGGE gel (Figure 5.2 and 5.5), illustrated another point. *L. monocytogenes* NCTC 11944 and ScottA, (which is also equivalent to *L. monocytogenes* 4b) was in fact the same strain that had a different designations or nomenclature. As far as *L. monocytogenes* NCTC 7973 is concerned, the DGGE technique was unable to differentiate this serotype from the rest.

With reference to the species of the genus *Listeria*, there was a distinct difference in the migratory pattern of *L. welshimeri*, *L. innocua* and *L. monocytogenes* (Figure 5.5). When agarose gel electrophoresis was used, a clear distinction between *L. monocytogenes* and *L. innocua* was not obtained since there was only a 15 bp difference between the two species. However, with DGGE analysis, a very clear distinction between the two species was obtained, making DGGE analysis more reliable compared to electrophoresis on agarose gels. No PCR product was obtained after amplification of the *iap* gene of *L. grayi* (Figure 5.4) and the result was confirmed by DGGE analysis (Figure 5.5). The primer set

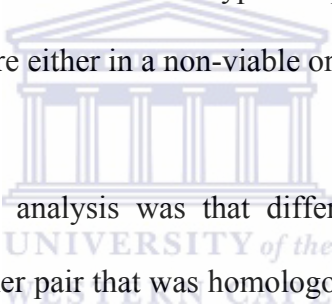
that was designed by Cocolin *et al.* (2002) was based on its partial alignment to the *iap* genes of *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seelgeri* and *L. ivanovii*. The primer pair was therefore not designed for the amplification of *L. grayi* as the results in this study confirm.

Food samples (ostrich meat and camembert cheese) spiked with *L. monocytogenes*, produced a positive result when subjected to agarose gel electrophoresis and the result was further confirmed by DGGE analysis. This molecular approach for pathogen detection has shown to be specific and sensitive enough for the isolation, detection and differentiation of *L. monocytogenes* in food products.



5.5 CONCLUSION

With the PCR-based DGGE technique, the identification and differentiation of *Listeria monocytogenes* from the other species of its genus was achieved as well as partial differentiation between *L. monocytogenes* serotypes. Of the *L. monocytogenes* serotypes used in this study, was only NCTC 4855 differentiated from the rest by DGGE analysis. Unfortunately, the ability of the DGGE technique to distinguish between serotypes of *L. monocytogenes* was only limited to a few serotypes employed in this study due to the unavailability of strains that were either in a non-viable or non-culturable state.



A major advantage of DGGE analysis was that differentiation at species level was achieved by using a single primer pair that was homologous to specific regions of the *iap* gene of the strains included in this study, generating a distinct migratory pattern for each strain on the polyacrylamide gel. Agarose gel electrophoresis analysis of PCR amplified sections of the *iap* gene were less distinctive; no differentiation between the serotypes of *L. monocytogenes* occurred as a 457 bp DNA fragment is a characteristic feature of all *L. monocytogenes* serotypes together with the fact that a 15 bp difference on agarose gels makes discrimination between *L. monocytogenes* and *L. innocua* less feasible. However, the evaluation of both agarose gel electrophoresis and DGGE makes interpretation of the results a lot more conclusive.

The protocol used for PCR-DGGE analysis firstly had to be optimized in order to make detection of *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seelgeri* and *L. ivanovii* more specific. Methods of DNA extraction, conditions for PCR amplification and DGGE analysis were all optimized in order to obtain a reproducible set of conditions that would enhance DNA amplification and augment the specificity and sensitivity of the protocol.

Conventional methods for the detection of *L. monocytogenes* was limiting since differentiation on selective media provided no distinction between *Listeria* spp. resulting in presumptive identification, whereas the PCR-DGGE method for *L. monocytogenes* identification in food products was sensitive and specific. Any inhibitory substances that may have been present in the ostrich and camembert cheese food samples had no negative effect on the sensitivity of the PCR-DGGE method. For future application, it is advisable to determine the detection limit of *L. monocytogenes* for PCR-DGGE analysis, to further verify the sensitivity and potential of this molecular technique.

The protocol for PCR-DGGE analysis of food samples in this study has proved to be reliable for food diagnostic purposes, especially since many conventional methods provide presumptive identification. The PCR-DGGE technique is a crucial parameter in the study of outbreaks of listeriosis and its application in this study allowed for the reliable monitoring of *Listeria* spp. and serotypes in food products and demonstrated the great potential that this method has over other techniques.

CHAPTER 6

6.1 CONCLUSION

Contamination of food products with *Listeria monocytogenes* occurs sporadically in South Africa. Although food-borne listeriosis is rare, the mortality rate is high among those at risk. From a public health viewpoint, there would be concern about the presence and numbers of *L. monocytogenes* in food products due to the ability of *L. monocytogenes* to cause food-borne disease and death. Furthermore, its ability to grow at refrigeration temperatures, which are temperatures used during storage in order to preserve and prevent spoilage of food products, makes it more of a threat to the human population and food industry. Isolation and detection methods that are more specific and robust and that would enhance the recovery rate and time of *L. monocytogenes* enumeration from food products is much desired in the food industry, as the economic implications of having to withdraw products that are suspected to have *L. monocytogenes* contamination has very huge repercussions.

The aim of this study was to optimize and implement sample preparation methods and molecular technology to lower the detection time of *L. monocytogenes* from food products and at the same time design a protocol that would be more specific and sensitive for the isolation and detection of *L. monocytogenes* in order to eliminate presumptive

positive and negative results. The methodology would thereby be validated in research and commercial laboratories.

Multiple factors including sample preparation methods, incubation times, DNA extraction methods and PCR constituents, which affect the sensitivity for the isolation of *L. monocytogenes* from food products, were evaluated in order to improve the sensitivity of the PCR assay. The United States Food and Drug Association (USFDA) policy stipulates that the sensitivity of an analytical method is actually 1 cfu 25 g⁻¹, although it has been questioned by the industry whether such low levels of *L. monocytogenes* are detectable in food products, since inhibitors present in food products may interfere with DNA amplification. The methods described in this study were able to detect 1 cfu 25 g⁻¹ *L. monocytogenes* in ostrich meat and camembert cheese samples. Primary enrichment of food samples in *Listeria* enrichment broth for 5 h, followed by a secondary enrichment in Fraser broth for 17 h was selected as the sample preparation method which allowed *L. monocytogenes* to recover and increase to a detectable limit. Optimization of PCR constituents resulted in an increase in the sensitivity of the PCR assay when both *Taq* and *Tth* DNA polymerase was used. The implementation of the optimized conditions for *L. monocytogenes* recovery from food products rendered an extremely low detection limit of 7-9 cfu ml⁻¹ in artificially contaminated camembert cheese, hake, minced meat and ostrich meat. It can be concluded that careful optimization of sample preparation methods and PCR constituents produced a very robust and reproducible method in order to attain a

detection limit of 7 cfu ml⁻¹ and 1 cfu 25 g⁻¹ *L. monocytogenes* in the food products tested.

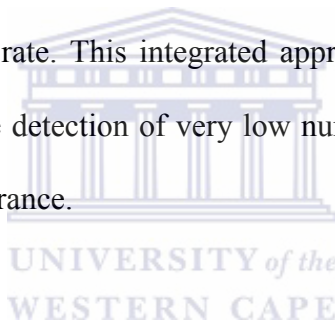
To further enhance the authenticity of the research undertaken, an internal amplification control (IAC) was constructed to co-amplify with *L. monocytogenes* in the PCR reaction. The inclusion of the IAC was to avoid false negatives, thus validating the potential of the PCR reaction as a powerful molecular tool for pathogen detection. A pre-determined region on the pUC19 genome was selected as the IAC. The amount of IAC was controlled and adjusted in order to avoid loss of target sensitivity. The concentration of pUC19 that was optimal for its co-amplification with *L. monocytogenes*, without having an inhibitory effect on the amplification of *L. monocytogenes*, was 0.001 pg µl⁻¹. Not only was the IAC successfully incorporated into the PCR reaction, but it had no negative effect on the detection limit of *L. monocytogenes* in food products. The method employed for *L. monocytogenes* isolation from food products was sensitive enough to detect 7-9 cfu ml⁻¹ and this detection limit was not compromised when an IAC was included in the reaction. Now that false negative PCR results can be correctly interpreted, the transition of the PCR reaction from research to commercial laboratories should occur with more ease. The construction of the IAC for *L. monocytogenes* detection is suitable to use in the food industry and would provide the assurance that negative PCR results are truly negative. The validity of PCR as a diagnostic tool for pathogen detection in food products was enhanced.

The PCR-DGGE method facilitated better distinction between *Listeria* spp. since a single base change between species was sufficient to produce a different migratory pattern on the polyacrylamide gel. The primer set that was used, was based on its partial alignment to the *iap* genes of *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. ivanovii*. Cultures presumed to be *L. seeligeri* and *L. ivanovii* were in fact identified as *L. monocytogenes* by DGGE analysis. Where distinction between *L. monocytogenes* and *L. innocua* was less pronounced on agarose gel electrophoresis, due to a difference of 15 bp, DGGE analysis was more effective in differentiating between the two species; which is important since *L. innocua* tends to mimic the appearance of *L. monocytogenes* and outgrow *L. monocytogenes* in a sample matrix. The PCR-DGGE method was suitable to differentiate between the genus *Listeria* and between the serotypes of *L. monocytogenes*. The implementation of DGGE will provide a better understanding of the ecology of food-borne pathogens in the food processing environment.

The methods used in this study highlighted the reliability and accuracy of molecular tools such as PCR and DGGE to provide critical information about the presence of *L. monocytogenes* in food processing environments. The information can be used to develop practical recommendations for the control of *L. monocytogenes* in the food industry as well as to form a standardized protocol for the detection of *L. monocytogenes* in both research and commercial laboratories. To further enhance the applicability of PCR-DGGE analysis, the detection limit for *L. monocytogenes* should be determined. It would

furthermore verify the sensitivity and potential of this method as a robust molecular approach for the detection and differentiation of *Listeria* in food products.

The cumulative effect of the methodology applied in this study forms the basis of a very accurate, sensitive and rapid pathogen detection system which is uncomplicated, yet very pertinent for future routine testing of food products in the food industry. A limitation relating to the usefulness of most isolation and detection methods is the time factor. The methods proposed in this study guarantees a very short detection time with a high sensitivity and reproducibility rate. This integrated approach for the enumeration of *L. monocytogenes* allowed for the detection of very low numbers of *L. monocytogenes* and would provide food safety assurance.



CHAPTER 7

7.1 REFERENCES

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Annexure 1

Listeria monocytogenes genome (Pubmed NCBI: accession number U25449) –
primer binding sites identified in bold.

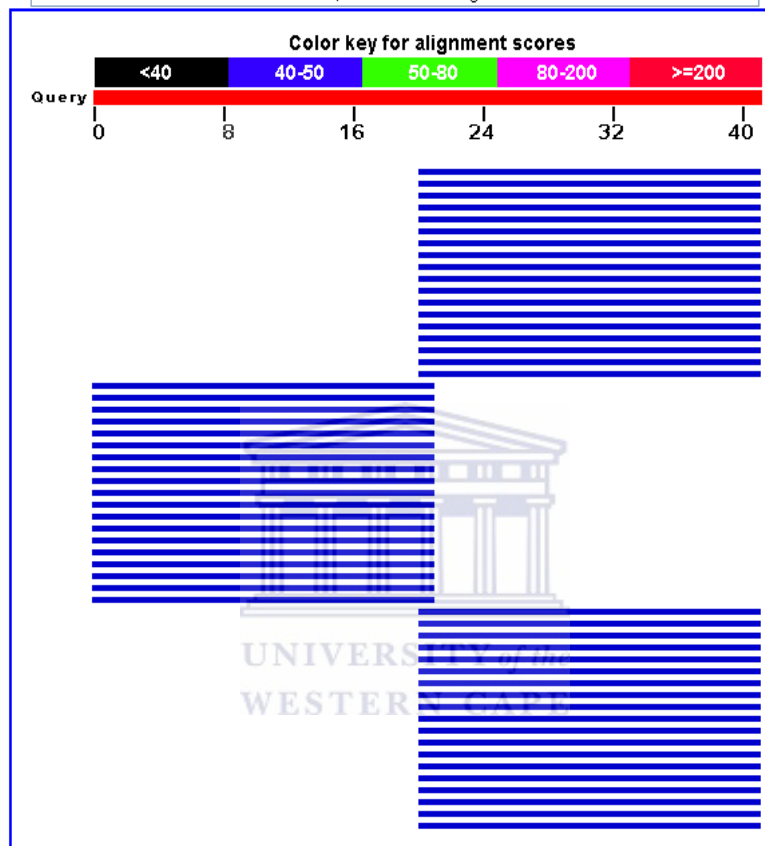
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Annexure 2

pUC 19 plasmid sequence (Pubmed NCBI: accession number L09137) – primer binding sites identified in bold.

```
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```

Annexure 3



The sequence of the forward primer (iacF) was blasted against all organisms and the profile above was the result. The results of the nucleotide-nucleotide BLAST indicating the alignment of the 5' end of iacF to *L. monocytogenes* and the IAC 3' end to various pUC19 cloning vectors.

Sequences producing significant alignments: (Bits) Value

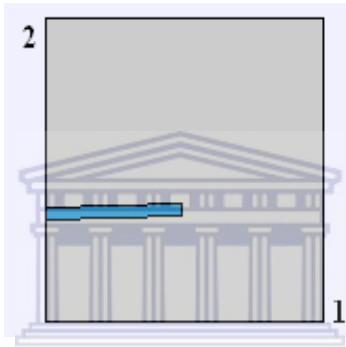
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0.081		
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0.081		
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0.081		

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gi 115336276 gb EF025088.1 0.081	Cloning vector pGT2, complete sequen	42.1
gi 114325529 gb DQ989355.1 0.081	Gene trapping Ds/T-DNA vector pDsG8,	42.1
gi 113204803 gb DQ489715.1 0.081	Plastid transformation vector pPRV31	42.1
gi 112941747 gb DQ842000.1 0.081	Promoter rescue plasmid pBK, complet	42.1
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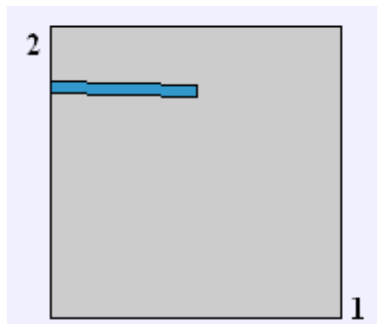
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 0.081
[gi|91521918|dbj|AB255647.1](#) | Exchangeable gene trap vector pU-... [42.1](#)
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 0.081
[gi|83779165|gb|DQ317600.1](#) | Positive selection cloning vector pJE [42.1](#)
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The alignment results of the forward primer (iacF) to the *L. monocytogenes* genome

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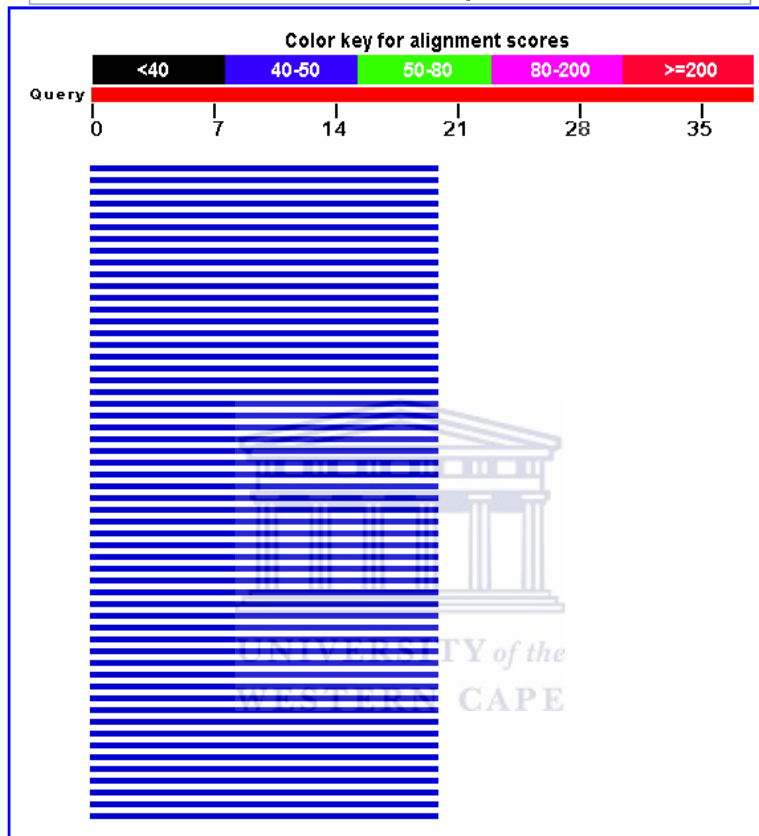


The alignment results of the reverse primer (iacR) to the *L. monocytogenes* genome

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Annexure 4



The sequence of the reverse primer (iacR) was blasted against all organisms and the profile above was the result. The results of the nucleotide-nucleotide BLAST indicating the alignment of the 5' end of the iacR primer to *L. monocytogenes*.

Sequences producing significant alignments:(Bits) Value

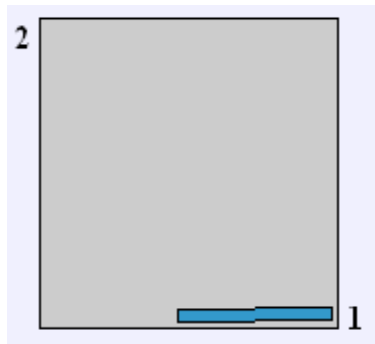
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gi 111182372 gb DQ838561.1 0.27	Listeria monocytogenes isolate 22...	40.1
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gi 111182342 gb DQ838551.1 0.27	Listeria monocytogenes isolate M1...	40.1
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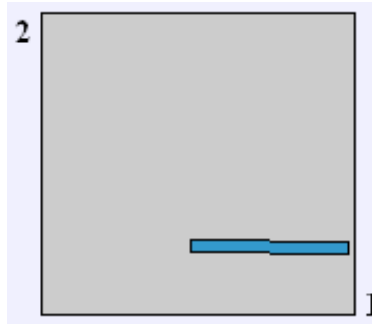


The alignment results of the forward primer (iacF) to the pUC19 genome

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