DETECTION AND MOLECULAR SUBTYPING OF *LISTERIA MONOCYTOGENES* ISOLATED FROM A SOUTH AFRICAN AVOCADO PROCESSING FACILITY

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

Listeria monocytogenes is a foodborne pathogen that has been isolated from a variety of food sources. It is the cause of the food-borne disease, listeriosis that shows symptoms such as meningitis, encephalitis and abortion. Different strains of *L. monocytogenes* exist and not all are thought to be pathogenic to humans. The aim of this study was to evaluate and compare conventional methods, culturing on selective (Oxford agar) and chromogenic (RAPID'*L.mono* agar) media, as well as species-specific and multiplex polymerase chain reaction (PCR) methods for the detection and identification of 94 *L. monocytogenes* isolates from various areas in an avocado processing facility, as well as the final product. To achieve a better understanding of the genetic diversity of the confirmed *L. monocytogenes* strains isolated from the avocado facility, two subtyping techniques, PCR-restriction fragment length polymorphism (PCR-RFLP) and pulsed-field gel electrophoresis (PFGE), were employed.

All of the isolates were identified as *Listeria* species on both Oxford and RAPID'*L.mono* agar. On the RAPID'*L.mono* agar, 76 of the 94 isolates produced colonies typical of *L. monocytogenes*, with the remaining 18 showing colonies typical of *L. innocua* (n=13) and *L. ivanovii* (n=5). The species-specific PCR successfully amplified a 730 base pairs region of the *hly* gene of 80 of the 94 isolates. For the same 80 isolates the multiplex PCR successfully amplified 800, 517 and 238 base pair (bp) fragments of the *inlA*, *inlC* and *inlJ* genes, respectively. The remaining 14 isolates included the 13 isolates identified as *L. innocua*, as well as an isolate identified as *L. monocytogenes* on RAPID'*L.mono*. The results obtained on the Oxford agar showed a 100 % positive correlation when compared to the PCR results in identifying *Listeria* species, while the RAPID'*L.mono* had a 4 % false negative result in identifying *L. monocytogenes* compared to the PCR results.

Sixty-four of the confirmed *L. monocytogenes* isolates were subtyped using PCR-RFLP and PFGE. For the PCR-RFLP analysis, a 733 bp fragment of the *inlA* gene was successfully amplified for all of the isolates, followed by digestion with the restriction enzymes, *Alu*I and *Tsp*509I. *Alu*I produced three different banding patterns and *Tsp*509I produced two different banding patterns. Subtyping of the isolates using PFGE was carried out by macrorestriction of the genomic DNA with *Apa*I and *Asc*I. The restriction fragments were resolved by PFGE and the fingerprints were classified into four clusters. In the combined analyses, cluster I contained forty-eight isolates (n=48),

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cluster II 1 isolate (n=1), cluster III fifteen isolates (n=15) and cluster IV 1 isolate (n=1). The PCR-RFLP results had a 98 % correlation with the PFGE results.

The results of this study indicated inconsistencies between the results obtained by conventional and molecular detection methods for the identification of *L. monocytogenes*. Species-specific and multiplex PCR, however, proved useful to accurately detect and identify *L. monocytogenes* in a shorter period of time and could replace the use of conventional agar during identification. Both PCR-RFLP and PFGE proved useful in the subtyping of *L. monocytogenes* isolates with the PCR-RFLP being less expensive and results obtainable in a shorter period of time.

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UITTREKSEL

Listeria monocytogenes is ‘n patogeen afkomstig van voedsel wat uit ‘n verskeidenheid voedselbronne geisoleer kan word. Dit is die oorsaak van die voedsel afkomstigde siekte, listeriosis met simptome soos harsingvliesontsteking, ensefalitis en aborsie. ‘n Verskeidenheid *L. monocytogenes* stamme bestaan, maar nie almal word as patogenies beskou nie. Die doel van hierdie studie was om konvensionele metodes, naamlik mikrobiologiese kweking op selektiewe (Oxford agar) en chromatografiese (RAPID’*L.mono* agar) media, sowel as spesies-spesifieke en multipleks polimerase ketting reaksie (PKR) metodes te evalueer en vergelyk vir die deteksie en identifikasie van 94 *L. monocytogenes* isolate geisoleer vanuit verskeie areas in ‘n avokado prosesseringsfasiliteit sowel as die finale produk. Om ‘n beter begrip van die genetiese diversiteit van die isolate wat as *L. monocytogenes* bevestig is te verkry, is twee subtiperingstegnieke, PKR-restriksiefragmentlengte polimorfisme (PKR-RFLP) en pulsveld jel-elektroforese (PVJE) toegepas.

Beide Oxford en RAPID’*L.mono* agar het al die isolate as *Listeria* spesies geidentifiseer. Op die RAPID’*L.mono* agar het 76 van die 94 isolate kolonies tipies van *L. monocytogenes* gevorm, 13 kolonies was tipies van *L. innocua* (n=13) en vyf kolonies tipies van *L. ivanovii* (n=5). Die spesies-spesifieke PKR het ‘n 730 basis paar (bp) streek van die *hly* geen suksesvol geamplifiseer vir 80 van die 94 isolate. Die multipleks PKR het 800, 517 en 238 bp fragmente van die *inlA*, *inlC* and *inlJ* gene onderskeidelik, vir dieselfde 80 isolate suksesvol geamplifiseer. Die oorblywende 14 isolate het die 13 isolate wat as *L. innocua* geïdentifiseer is en die een isolaat wat as *L. monocytogenes* op RAPID’*L.mono* geïdentifiseer is ingesluit. Resultate verkry met die Oxford agar het 100 % ooreengestem met die PKR resultate vir die identifikasie van *Listeria* spesies. Die RAPID’*L.mono* het ‘n 4 % vals negatiewe resultaat gelewer in vergelyking met die PKR resultate.

Vier-en-sestig van die bevestigde *L. monocytogenes* isolate is gesubtipeer deur PKR-RFLP en PVJE. Tydens die PKR-RFLP analise is ‘n 733 bp fragment van die *inlA* geen suksesvol geamplifiseer, gevolg deur vertering met die restriksie-ensieme, *Alu*I and *Tsp*509I. *Alu*I het drie verskillende bandpatrone opgelewer en *Tsp*509I twee verskillende bandpatrone. Subtipering deur PVJE is uitgevoer deur makro-restriksie van die genomiese DNA met *Apa*I en *Asc*I. Die restriksie fragmente is geskei deur PVJE en die vingerafdrukke is in vier groepe geklassifiseer. Groep I het 48 isolate (n=48), groep II 1 isolaat (n=1), groep III 15 isolate (n=15) en groep IV 1 isolaat (n=1)

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gehad tydens die gekombineerde analise. Die PKR-RFLP resultate het 98 % ooreengestem met die van die PVJE.

Die resultate van hierdie studie het teenstrydighede tussen die resultate van konvensionele en molekulêre deteksie metodes opgelewer vir die identifikasie van *L. monocytogenes*. Die spesies-spesifieke en multipleks PKR het egter beide goed te pas gekom vir die akkurate deteksie en identifikasie van *L. monocytogenes* en kan heel moontlik die gebruik van konvensionele agar tydens identifikasie vervang. Beide PKR-RFLP en PVJE was nuttig vir die subtipering van *L. monocytogenes* isolate. PKR-RFLP is egter ‘n goedkoper tegniek en die resultate is in ‘n korter tydsperiode beskikbaar.

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The important thing is not to stop questioning.

Curiosity has its own reason for existing.

Albert Einstein

Dedicated to my parents

Johan en Cecile Bester

I thank you and I love you

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Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

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CHAPTER 1

INTRODUCTION

Until the 16th century, the avocado was known only to the inhabitants of Mexico, Central America and northern parts of South America, where it originated. The Spanish Conquistadors were the first foreigners to discover the avocado and initiated the distribution of the avocado seed to the rest of the world. The plant was introduced to South Africa during the late 19th century and despite a slow initial growth phase has led to a very profitable industry (Van Zyl & Ferreira, 1995).

Presently, the South African avocado industry is a multi-million rand industry with an estimated gross value of R297 534 000 for avocados produced during the 2006/2007 season (Anon., 2009). Avocados exported from South Africa are mostly destined for Europe supplying 21 % of its total imports (Van Zyl & Ferreira, 1995). Processing of avocados for guacamole in South Africa started in 1998 with 250 tonnage raw product and has since increased to 6 500 tonnage raw product in 2007 (Anon., 2010). Guacamole is consumed locally and also exported and, therefore, has to adhere to stringent laws set by international trade regulations. The *Listeria monocytogenes* microbial load of guacamole is especially important as the United States of America has a zero tolerance policy and Canada and Europe a 100 cfu g^{-1} limit in ready-to-eat foods (EC, 1999; McLauchlin *et al*., 2004).

Listeria monocytogenes has only recently emerged as a food-borne pathogen with the first confirmed case in 1981 linked to contaminated coleslaw. It is responsible for the disease listeriosis, which can cause severe malaise in pregnant women, neonates, the elderly and persons suffering from a weakened immune system. Without treatment, listeriosis can develop into septicaemia and meningitis, as well as abortion and stillbirths (WHO, 2002; Montville & Matthews, 2008). With a mortality rate fast approaching 30 %, it is considered to exceed those of common food-borne pathogens such as *Salmonella enteritidis*, *Campylobacter* species and *Vibrio* species (Altekruse *et al*., 1997; Mead *et al*., 1999; Montville & Matthews, 2008). *Listeria monocytogenes* is most commonly found in raw or processed dairy, meat, vegetables and seafood products and especially products that are kept at refrigeration temperature as *L. monocytogenes* can grow at these low temperatures (Meng & Doyle, 1997; Gugnani, 1999; WHO, 2002).

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The most common methods used to detect *L. monocytogenes* are conventional methods that rely on the use of microbiological media to selectively grow and enumerate this micro-organism. These methods are inexpensive, sensitive and provide results that are both qualitative and quantitative. These methods are, however, time-consuming and labour-intensive (De Boer & Beumer, 1999). The approved methods that are currently been using for the detection of *L. monocytogenes* in foods include the Federal Drug Administration (FDA) protocol, ISO-11290-1, Netherlands Government Food Inspection Service (NGFIS) and US Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) methods (Churchill *et al*., 2006).

Recent advances in molecular genetics have led to methods targeting unique genes for amplification and subsequent differentiation. Therefore, using a unique gene, *L. monocytogenes* can be distinguished from other *Listeria* species. These methods are intrinsically more accurate and less affected by natural variation than conventional methods and offers high specificity, sensitivity and a rapid turnover that is essential for producers of minimally processed food products (Liu, 2006).

Furthermore, the incidence of listeriosis cases in South Africa is unknown as no system for reporting cases are in place. Information regarding the prevalence and genetic diversity of this micro-organism in South Africa is also limited. As a large percentage of the South African population is HIV-positive or immuno-compromised due to tuberculosis, it is essential that further research on this micro-organism be conducted.

The aim of this study was to evaluate and compare various methods for the detection and identification of *L. monocytogenes* isolates from various areas in an avocado processing facility, as well as the final product. These included conventional methods, culturing on selective and chromogenic media, as well as species-specific and multiplex polymerase chain reaction (PCR). To achieve a better understanding of the genetic diversity of the confirmed *L. monocytogenes* strains isolated from the avocado facility, two subtyping techniques, restriction fragment length polymorphism (RFLP) and pulsed-field gel electrophoresis (PFGE) were employed.

References

Altekruse, S.F., Cohen, M.L. & Swerdlow, D.L. (1997). Emerging foodborne diseases. *Emerging Infectious Diseases*, **3**, 285–293.

Anonymous (2009). *Abstract of Agricultural Statistics 2009*. Pretoria: Department of Agriculture.

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Anonymous (2010). Industry statistics. [WWW document]. URL http://www.subtrop.net/home.html. 15 February 2010.

Churchill, R.L.T., Lee, H. & Hall, J.C. (2006). Detection of *Listeria monocytogenes* and the toxin listeriolysin O in food. *Journal of Microbiological Methods*, **64**, 141–170.

De Boer, E. & Beumer, R.R. (1999). Methodology for detection and typing of foodborne microorganisms. *International Journal of Food Microbiology*, **50**, 119–130.

European Commission (1999). *Opinion of the Scientific Committee on Veterinary Measures Relating to Public Health on Listeria monocytogenes*. European Commission, Health and Consumer Protection Directorate General, Brussels, Belgium.

Gugnani, H.C. (1999). Some emerging food and water borne pathogens. *Journal of Communicable Diseases*, **31**, 65–72.

Liu, D. (2006). Identification, subtyping and virulence determination of *Listeria monocytogenes*, an important foodborne pathogen. *Journal of Medical Microbiology*, **55**, 645–659.

McLauchlin, J., Mitchell, R.T., Smerdon, W.J. & Jewell, K. (2004). *Listeria monocytogenes* and listeriosis: a review of hazard characterization for use in microbiological assessment of foods. *International Journal of Food Microbiology,* **92**, 15–33.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M. & Tauxe, R.V. (1999). Food-related illness and death in the United States. *Emerging Infectious Diseases*, **5**, 607–625.

Meng, J. & Doyle, M.P. (1997). Emerging issues in microbiological food safety. *Annual Review of Nutrition*, **17**, 255–275.

Montville, T.J. & Matthews, K.R. (2008). *Listeria monocytogenes*. In: *Food Microbiology, An Introduction*, 2nd ed. Pp 173–187. Washington, DC: ASM Press.

Van Zyl, J.L. & Ferreira, S.G. (1995). An overview of the Avocado Industry in South Africa. *South African Avocado Growers' Association Yearbook*, **18**, 23–30.

World Health Organisation (WHO) (2002). Foodborne diseases, emerging. Fact Sheet No. 124. [WWW document]. URL http://www.who.int/mediacentre/factsheets/fs124/en/. 21 October 2010.

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CHAPTER 2

LITERATURE REVIEW

A. Background

The history of the avocado in South Africa is relatively recent, with the first avocado being introduced by the Dutch colonists. These original avocados were imported from the West Indian Islands, but during the 1920s improved planting material were imported from Mexico, Guatemala and California, which launched the South African avocado industry. During the 1930s, the number of avocado trees was approximately 10 000 and the first commercial orchard was planted in KwaZulu-Natal. Since 1965, avocado production has increased with an average growth rate of 8 % per annum. This led to the founding of The South African Avocado Growers' Association in 1967 to regulate exports in order to stabilise prices. Today, approximately 550 farmers are involved in avocado production with farming units varying from 20 to 30 hectares (Van Zyl & Ferreira, 1995). The total population of avocado trees in South Africa has increased to 3 015 000, with 90 % of the trees located in Limpopo and Mpumalanga (Anon., 2010a). The most popular cultivars in South Africa include Fuerte, Hass, Ryan, Pinkerton, and Edranol. The harvesting season for the two most popular cultivars, Fuerte and Hass is from March to September, with the other cultivars being harvested during the rest of the year. Most of the world's exported avocados are traded in Europe. Israel is the main exporter to Europe and supplies 29 % of the total imports. South Africa is the second main exporter supplying 21 % and Spain is third with 17 % (Van Zyl & Ferreira, 1995).

Since 1970, the total tonnage of avocados produced in South Africa has increased from 1 480 to 93 027 in 2007 and the total tonnage of avocados exported has increased from 1 480 to 51 080 during the same period, with the balance being consumed locally. Processing of avocados for guacamole started in 1998 with 250 tonnage raw product and has increased to 6 500 tonnage raw product in 2007 (Anon., 2010a). The estimated gross value of avocados produced during the 2006/2007 season was R297 534 000 (Anon., 2009).

B. Listeria

The history of *Listeria* and *Listeria monocytogenes* is relatively recent with the first published description of the bacteria by Murray *et al*. in 1926. Previous to this report, a few reports may have described *Listeria* isolations, but the isolates were never

deposited into permanent collections and could subsequently not be further investigated (Rocourt, 1961). Up to the 1970s, the relationship of *Listeria* to other bacteria remained unclear. These bacteria were included in the fourth edition of *Bergey's Manual of Determinative Bacteriology* and were included in the Corynebacteriaceae family under the genus *Kurthia*. In the 1974 edition of *Bergey's Manual of Determinative Bacteriology,* the genus *Listeria* was moved to the Lactobacillaceae family.

Based on morphological resemblances, *Listeria* was associated with the coryneform group of bacteria, being Gram-positive and a non-endospore-forming rod. However, with the development and introduction of new methods and technologies such as numerical taxonomy, chemotaxonomy, DNA/DNA hybridation and ribosomal RNA (rRNA) gene sequencing, the phylogenetic position of *Listeria* has become clearer. During the last three decades, information and data was accumulated and *Listeria* is now a well-defined taxon with distinctive characteristics. It is not a coryneform bacteria, as previously thought, having a low G+C % DNA content, absence of mycolic acids, and the presence of lipoteichoic acid (Rocourt, 1999).

The genus *Listeria* was for many years monospecific containing only *L. monocytogenes*, the type species. *Listeria denitrificans*, *L. grayi*, *L. murrayi*, *L. innocua*, *L. welshimeri, L. seeligeri* and *L. ivanovii* was added to the genus in 1948, 1966, 1971, 1981, 1983, 1983 and 1985, respectively (Sohier *et al*., 1948; Larsen & Seeliger, 1966; Welshimer & Meredith, 1971; Seeliger, 1981; Rocourt & Grimont, 1983; Seeliger *et al*., 1984). With the introduction of molecular methods, the diversity within the genus *Listeria* is better understood and now only contains the six species *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri* and *L. grayi* (Rocourt, 1999). Based on sequencing data of the 16S and 23S rRNA genes, the genus can be divided into two directly related, but distinct lines of descent. The one line contains *L. grayi* and the other line the five remaining species (Sallen *et al*., 1996). Among these six species, *L. monocytogenes* is pathogenic to humans and animals, *L. ivanovii* has two subspecies *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis* and is pathogenic to animals and the remaining four species are considered to be non-pathogenic. *Listeria seeligeri* is considered to be non-pathogenic, but has a part of the virulence gene cluster which is present in the two pathogenic species, *L. monocytogenes* and *L. ivanovii* (Bhunia, 2008).

The *Listeria* virulence gene cluster, which comprises six coherent chromosomal genes all physically linked in a 9 kb chromosomal island, is referred to as the *Listeria* pathogenicity island 1 (LIPI-1) (Fig. 1) (Vazquez-Boland *et al*., 2001). LIPI-1 includes

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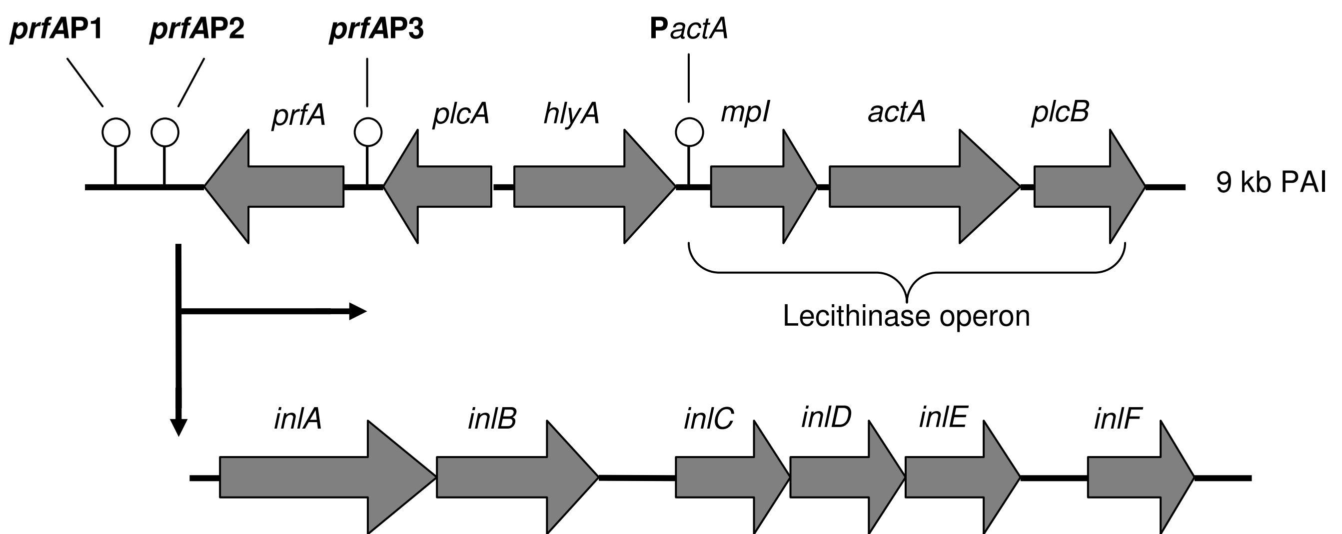

Figure 1 The virulence gene clusters in the 9 kb pathogenicity island (LIPI-1) of *Listeria monocytogenes.* The internalin gene family is also shown. Open circles represent promoters (adapted from Bhunia, 2008).

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the genes *prfA* (encodes positive regulatory factor A), *plcA* (phosphatidylinositol phospholipase C (PI-PLC)), *hlyA* (haemolysin listeriolysin O (LLO)), *mpl* (zinc metalloprotease), *actA* (surface actin polymerisation protein) and *plcB* (phosphatidylcholine phospholipase C (PC-PLC)). The latter gene can be used to distinguish between the different *Listeria* species as it is only present in *L. ivanovii, L. seeligeri* and *L. monocytogenes* (Gouin *et al*., 1994; Bhunia, 2008). The gene *plcA,* which encodes PI-PLC (33 – 36 kDa), however, is only present in *L. ivanovii* and *L. monocytogenes*. PI-PLC and LLO acts in synergy to destroy the phagosome in which the bacterium is trapped during infection. This is one of four major steps involved in the cellular mechanism of *Listeria* pathogenesis. The absence of the *plcA* gene in *L. seeligeri* is, therefore, a contributing factor to its non-pathogenic status (Bhunia, 2008).

The morphology of *Listeria* can be described as a small (1 - 2 µm in length and 0.5 µm in diameter), regular, Gram-positive rod with rounded ends. The cells can be found alone or in groups, including short chains or V and Y arranged forms depending on the growth conditions and temperature. They do not produce spores and do not form capsules. The cells might sometimes be coccoid and when older, lose the ability to retain Gram stain. The bacterial cells can then be mistaken for streptococci or *Haemophilus* spp. (Jorgensen *et al*., 1995; Bhunia, 2008). When cultured at 20 ° - 25 °C, *Listeria* is motile due to the formation of a few peritrichous flagella. These flagella are very weak or absent when *Listeria* is cultured at 37 °C (Galsworthy *et al*., 1990).

Listeria grows well on standard bacteriological agar. When grown on nutrient agar, colonies that are 0.2 - 0.8 mm in diameter, smooth, translucent, punctiform, bluish gray and slightly raised can be observed after 24 h of incubation. After 5 - 10 days of incubation, colonies will be well separated and may be 5 mm in diameter. The growth rate of *Listeria* can be increased by the presence of glucose, a fermentable sugar (McKellar, 1994). When grown in broth, the medium becomes cloudy after 8 - 24 h of incubation at 37 °C. *Listeria* grows better at oxygen tensions just lower than that of air, as indicated by the profuse growth that can be observed just below the clear area near the surface of the medium. *Listeria* can be classified as aerobic, facultatively anaerobic, microaerophilic, catalase-positive and oxidase-negative (Seeliger, 1961).

Listeria species are psychrophilic and can grow at temperatures ranging from 0 ° - 45 °C, however, growth below 0 °C has been reported (Junttila *et al*., 1988; Walker *et al*., 1990). Growth can occur within the pH range of 4.4 - 9.6, but optimal growth is observed at pH 7 (George & Lund, 1992). At pH values below 4.3, cells do not grow but

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may survive (Montville & Matthews, 2008). *Listeria* cells can also tolerate a 10 % (w/v) NaCl solution and survive at even higher concentrations (Seeliger & Jones, 1986). The survival at high salt concentrations and low pH is strongly temperature dependent (Cole *et al*., 1990). *Listeria* species grows best at water activity (a_w) values ≥ 0.97, but some strains can also grow at an a_w value below 0.93, which is rare for foodborne pathogens. *Listeria* species may even survive at a_w values as low as 0.83 for long periods of time. It has also been shown that the heat resistance of *Listeria* species increases as the a_w decreases (Farber *et al*., 1992; Montville & Matthews, 2008). Having these extreme tolerances regarding pH, temperature, water activity and salt conditions (Sleator *et al*., 2003; Liu *et al*., 2005) makes the survival of this genus in a variety of environments possible. Soil, water, effluents and food has been identified as suitable environments for the survival and subsequent isolation of *Listeria* species (Liu, 2006).

As *Listeria* species exhibit similar morphological, serological and biochemical characteristics, as well as a degree of variation in test results between isolates of the same species, a laborious testing scheme is needed to correctly differentiate between the species (Vazquez-Boland *et al*., 2001). This testing scheme involves *in vitro* culturing with enrichment and selective media, as well as several subsequent morphological, serological and biochemical tests (Robinson *et al*., 2000). Besides being time-consuming (results taking up to 6 days), the tests are also based on phenotypic characteristics of *Listeria* that can give variable results (Aznar & Alarcon, 2002). A study by Aznar and Alarcon (2002) demonstrated that identification of *Listeria* species by biochemical tests is not always accurate and that genetic testing has led to the reclassification of some isolates. Genetic testing is less likely to be influenced by external factors that could alter the metabolism and growth of the bacteria, as they detect differences at the genetic level (Batt, 1997). Molecular methods are, therefore, intrinsically more specific and sensitive for the identification and differentiation of *Listeria* species.

Genetic tests that have been used for the detection and/or identification of *Listeria* species have exploited the molecular differences of the 16S and 23S rRNA genes (Graham *et al*., 1996, 1997; Sallen *et al*., 1996). Polymerase chain reaction assays have also been developed to target specific virulence genes such as the *hlyA* gene (part of LIPI-1) which is present in only certain *Listeria* species (Blais *et al*., 1997) and the *iap* gene, which is a virulence gene common to all *Listeria* species (Bubert *et al*., 1999).

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C. Listeria monocytogenes

Listeria monocytogenes is a ubiquitous Gram-positive, psychrotrophic (De Oliveira *et al*., 2010), foodborne pathogen (Salamina *et al*., 1996; Aureli *et al*., 2000; Sim *et al*., 2002) that is the known cause of listeriosis (De Vasconcelos *et al*., 2008). It is widely distributed in nature and is commonly found in soils, decaying vegetation, sewage, silage, animal faeces and fresh and salt water (Jay, 1996). It can also be found in the human body and in healthy humans. One to five percent of the world's population is reported to serve as carriers of these bacteria (Bhunia, 2008). In foods, the bacteria has been detected in both raw and processed foods including meat, seafood, dairy products and vegetables (Meng & Doyle, 1997; Gugnani, 1999). Due to the lack of good agricultural practices, vegetables are especially prone to microbiological contamination by the use of untreated water or contaminated organic fertilisers (Francis *et al*., 1999).

Listeria monocytogenes contains 13 distinct serovars (O-antigenic patterns) 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, 4ab and 7 (Bhunia, 2008). With the use of various subtyping techniques, including serotyping, *L. monocytogenes* has been divided into three genetic lineages. Lineage I contains the serotypes 1/2b, 3b, 3c and 4b. Lineage II contains serotypes 1/2a, 1/2c and 3a and Lineage III contains the serotypes 4a and 4c. Isolates belonging to the serotypes in lineage I include clones of *L. monocytogenes* which are responsible for human listeriosis outbreaks and has the highest pathogenic potential (Saunders *et al*., 2006; Bhunia, 2008). Those of lineage II have intermediate pathogenic potential and are usually isolated from foods and the environment and those from lineage III have a low pathogenic risk, usually found in animal hosts and rarely cause human infections (Kathariou, 2002; Bhunia, 2008). Serovars 1/2a (lineage II), 1/2b and 4b (both lineage I) have been identified as the serovars responsible for the majority of listeriosis cases (Vines & Swaminathan, 1998; De Vasconcelos *et al*., 2008). Doumith *et al*. (2004b) interestingly found that while serovar 4b, from lineage I, which is considered the most virulent and responsible for the majority of epidemic listeriosis cases, it was in fact serovar 1/2a, from lineage II, that was most frequently isolated from contaminated food (Doumith *et al*., 2004b). This result was in agreement with reports by Ericsson *et al*. (1995), Kathariou (2002), Gray *et al*. (2004), Jacquet *et al*. (2004) and Ramaswamy *et al*. (2007) and may indicate that not all food contaminated with *L. monocytogenes* will evoke a listeriosis epidemic.

Listeria monocytogenes is pathogenic at the species level, but various strains display varied virulence and pathogenic potential. There are strains that may be very

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virulent and cause disease and others that are non-pathogenic and produce no apparent malaise (Erdenlig *et al*., 2000; Gracieux *et al*., 2003; Liu *et al*., 2003b; Liu, 2004; Doumith *et al*., 2004b). The difference between these virulent and non-virulent strains is minimal as they possess the same virulence gene cluster LIPI-1 (consisting of *prf*A, *hly*A, *plc*A, *mp*l, *act*A, and *plc*B), and all of the major virulence proteins involved in *L. monocytogenes* pathogenesis, but may differ in the expression of these genes (Liu *et al*., 2003a).

The cellular mechanism of *L. monocytogenes* pathogenesis can be divided into four major steps, namely adhesion and invasion, lysis of vacuoles, intracellular growth and cell-to-cell spread (Fig. 2) (Bhunia, 2008). In each of these steps there are various virulence proteins that are involved and necessary for *Listeria* pathogenesis. A number of adhesion factors have been identified to be involved in the adhesion of the bacterium to the host cell, namely internalin A (InlA), internalin B (InlB), virulence-associated invasion protein (Vip), *Listeria* adhesion protein (LAP), fibronectin-binding protein (Fbp), autolysin amidase (Ami), cell wall hydrolase (p60), lipoprotein promoting entry (LpeA) and lipoteichoic acid (LTA). The size, receptor and function of each of these virulence proteins are indicated in Table 1 (Bhunia, 2008). The size of Ami varies among the *L. monocytogenes* serotypes. In serotype 4b, it is 770 amino acids long whereas in serotype 1/2a it is 917 amino acids long (Bhunia, 2008).

After the bacterium has attached and entered the host cell, it is trapped inside a vacuole (phagosome). Two virulence proteins have been identified that destroys the phagosome and allows the bacterium to escape, namely LLO and PI-PLC (Bhunia, 2008). LLO, encoded by the *hlyA* gene (part of LIPI-1), is responsible for the haemolysis of blood cells and the disruption of eukaryote membranes (Kingdon & Sword, 1970). LLO is a sulfhydryl (SH)-activated hemolysin with a molecular mass of 58 – 60 kDa (Bhunia, 2008). The function of LLO, a bacterial pore-forming hemolysin, is to lyse the membranes of phagosomal vesicles and to facilitate the escape of *L. monocytogenes* into the cytoplasm of the cell (Decatur & Portnoy, 2000; Kalpana *et al*., 2004). The maximum cytolytic activity of LLO coincides with the phagosomal pH value of 5.5 (Bhunia, 2008). LLO is essential for virulence by *L. monocytogenes* and is only found in virulent strains. As LLO is a secreted protein, the detection of it in a food sample can also be an indicator of the presence of *L. monocytogenes* cells (Vazquez-Boland *et al*., 2001). PI-PLC, encoded by the *plcA* gene (part of LIPI-1), acts synergistically with LLO to destroy the lipid bilayer of the phagosome. Both of the genes that encode for LLO and PI-PLC, *hly* and *plcA*, respectively are regulated by *prfA*

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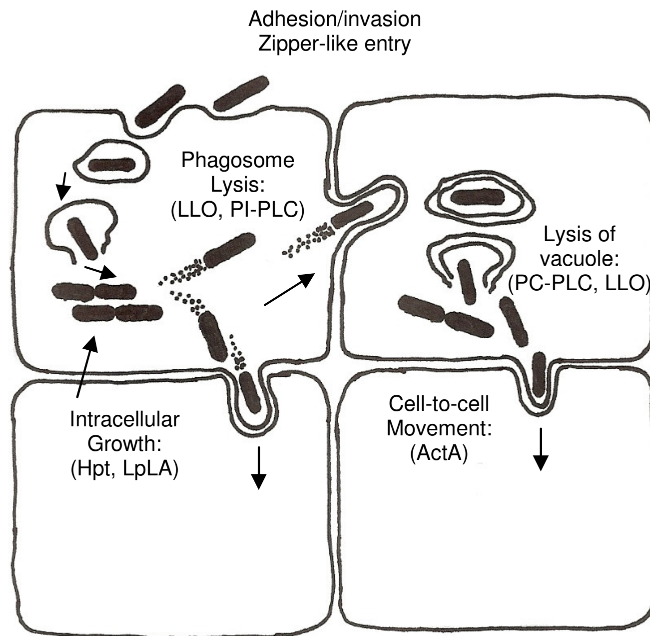

Figure 2 Cellular mechanism of *Listeria monocytogenes* pathogenesis (adapted from Bhunia, 2008).

Table 1 Major virulence proteins in *Listeria monocytogenes* (Adapted from Bhunia, 2008).

Virulence factors	Size (kDa)	Receptor	Function
Protein regulatory factor (PrfA)	27	-	Regulation of virulence protein expression.
Internalin A (InlA)	88	E-cadherin (tight junction protein)	Responsible for invasion into intestinal epithelial cells and placenta during pregnancy.
Internalin B (InlB)	65	Met (tyrosine kinase), gClq-R/p32	Entry into hepatocytes and hepatic phase of infection.
Virulence protein (Vip)	43	Gp96 (chaperone protein)	Invasion of epithelial cells.
Listeria adhesion protein (LAP)	104	Hsp60 (chaperone protein)	Adhesion to intestinal epithelial cells.
Autolysin amidase (Ami)	102	Peptidoglycan	Adhesion to host cells.
p60 (cell wall hydrolase)	60	Peptidoglycan	Adhesion/invasion.
Listeriolysin (LLO)	58 – 60	Cholesterol	A hemolysin aids in bacterial escape from phagosome inside the cell.
Actin polymerisation protein (ActA)	90	-	Nucleation of actin tail for bacterial movement inside the cytoplasm.
Phospholipase (*plcA* – PI-PLC; *plcB* – PC-PLC)	29 – 33	-	Lyses of vacuole membrane
Metalloprotease (Mpl)	29	-	Helps synthesis of PLC.

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(also part of LIPI-1). A mutation in the *prfA* gene will result in the inactivation of all the genes located downstream from it, including *hly* and *plcA*. These two genes are essential for the maintenance of the virulent status of *L. monocytogenes* (Bhunia, 2008) and this mutation might be the reason for the existence of non-virulent *L. monocytogenes* strains.

After the bacterium has escaped from the phagosome, it first multiplies before moving into a new cell. Although several protein factors are involved during this step, they are not regarded as major virulence proteins. Hexose phosphate translocase (Hpt) and lipoate protein ligase (LpLA1) are expressed to utilise host-derived glucose and lipoic acid for multiplication (Bhunia, 2008). After multiplication, the bacteria spreads to new cells. Three virulence proteins have been identified which aids in this spread, namely actin polymerisation protein (ActA), PC-PLC and zinc metalloprotease (Mpl). All three of the genes that encode for ActA, PC-PLC and Mpl, namely *actA, plcB* and *mpl*, are part of LIPI-1 and regulated by *prfA* (also part of LIPI-1). A mutation in the *prfA* gene will result in the inactivation of all the genes located downstream from it, including *actA, plcB* and *mpl*. A mutation in the *actA* gene itself will lead to mutant strains that are unable to accumulate actin and, therefore, unable to infect adjacent cells. A mutation in either the *plcB* or *mpl* genes will also lead to reduced virulence (Bhunia, 2008).

An important factor to consider is the expression of the genes that encodes major virulence proteins. Spesialised DNA binding proteins or transcriptional regulators play an essential role in the adaptation and survival of bacteria in different environmental conditions by directing gene expression. Different bacterial species and subspecies have the ability to adapt to different and sometimes highly specialised environmental conditions and, therefore, each bacterial group possesses these unique transcriptional regulators. This characteristic may be used for diagnostic applications as the transcriptional regulators may be genus-, species-, or subspecies-specific (Liu *et al.*, 2003a) and could be used to differentiate between the virulent and non-virulent *L. monocytogenes* strains.

Some strains of *L. monocytogenes* have the ability to adapt to food processing environments and persist in this environment for up to ten years, due to the formation of biofilms (Kathariou, 2002; Tompkin, 2002). The formation of biofilms, where *L. monocytogenes* cells adsorb to inert surfaces, is well known and documented (Mafu *et al.*, 1990; Hood & Zottola, 1997; Kalmokoff *et al.*, 2001). Thick, complex biofilms are more resistant to stress and difficult to remove or disinfect (using sanitising agents and

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antimicrobials) than adhered single cells (Lappi *et al*., 2004). A study by Pan *et al.* (2006) who investigated the resistance of *L. monocytogenes* biofilms to sanitising agents, primarily used serotype 4b strains, but several studies have reported that there is a limited correlation between the serotype and the ability to form biofilms (Kalmokoff *et al*., 2001; Borucki *et al*., 2003; Doumith *et al*., 2004a). It has, however, been demonstrated that several factors including physical and chemical properties of the substrate for attachment, characteristics of the strain, growth phase of the bacteria, growth media, temperature and the presence of other micro-organisms influence the cell attachment and biofilm formation ability of *L. monocytogenes* (Mafu *et al*., 1991; Blackman & Frank; 1996; Wong, 1998; Briandet *et al*., 1999; Norwood & Gilmour, 2000; Kalmokoff *et al*., 2001; Norwood & Gilmour, 2001; Chavant *et al*., 2002).

D. Listeriosis

The first confirmed case of listeriosis in a human was diagnosed at the end of World War I from a soldier suffering from meningitis. It was confirmed that the bacterium responsible for the disease was *L. monocytogenes* by retrospective identification of the strain (Rocourt, 1961). Infection with *L. monocytogenes* is neither population nor gender specific and is infective to all human population groups. The most susceptible/highest risk group includes pregnant women, neonates, the elderly and immuno-suppressed individuals such as those with HIV/Aids, cancer patients receiving chemotherapy or patients receiving treatment for organ transplantation (Vazquez-Boland *et al*., 2001). The initial symptoms of human listeriosis include fatigue, chills, headache, and muscular and joint pain (non-specific flu-like symptoms) and also gastro-enteritis. Without treatment, the disease can develop into meningitis, encephalitis, septicaemia, abortion and death (Vazquez-Boland *et al*., 2001).

Listeria monocytogenes has a range of virulence factors that enables it to cross the cerebrospinal, intestinal and placental barriers. The organism escapes the human immune surveillance system by internalisation (moving into the cells) and then multiplying in the cytosol of infected cells (Wiedmann *et al*., 1997). Pregnant women infected with *L. monocytogenes* may transfer the disease to their fetuses, which could lead to abortion, stillbirth or the premature birth of an infected child (Seeliger & Jones, 1986; Spencer, 1987).

The mortality rate of infection with *L. monocytogenes* is approaching 30 %, which indicates a high fatality:case ratio and exceeds those of common foodborne pathogens such as *Salmonella enteritidis*, *Campylobacter* spp. and *Vibrio* spp. (Altekruse *et al*.,

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1997; Mead *et al.*, 1999; Montville & Matthews, 2008). The infectious dose of *L. monocytogenes* is still unknown, but depends on the virulence of the microbe, the immunological status of the human and the contaminated food. Studies with test animals indicated that by reducing the exposure levels reduces the incidence of clinical disease. Foods responsible for serious outbreaks all had >100 cfu g^{-1} *L. monocytogenes*, however, more epidemiologic data is needed to accurately determine the infectious dose (McLauchlin *et al.*, 2004; Montville & Matthews, 2008).

As the infectious dose is still unknown, the official regulations regarding *L. monocytogenes* vary among food products, as well as countries. Regulations range from a "zero tolerance" level (absence of *L. monocytogenes* in 25 g of food sample) in the United States issued by the Food and Drug Administration (FDA) for Ready-to-eat (RTE) foods (Chen *et al.*, 2003), to an absence in 1 g in Europe, and to less than 100 cfu g^{-1} in RTE foods that do not support the growth of the pathogen at the time of consumption, also in Europe (EC, 1999; EC, 2000; Anon., 2005).

These limits represents a serious challenge to the food industry. Foods of particular concern are those that do not receive a heat inactivation step during processing (Gombas *et al.*, 2003; Thimothe *et al.*, 2004) and are stored at refrigeration temperatures. Contamination of food post-process represents a severe problem because of the ability of *L. monocytogenes* to survive and grow at these refrigeration temperatures (Faber & Peterkin, 1991). A study by Liu *et al.* (2004) concluded that acid, alkali and/or salt treatments used in some food processing plants as hurdle technology to eliminate *L. monocytogenes* may not be adequate due to the extreme environmental tolerances of this organism. To ensure products free of contamination, stringent quality control measures needs to be in place at the beginning, as well as at the end of the production process to avoid a recall of contaminated food and a possible listeriosis epidemic (Churchill *et al.*, 2006).

Listeriosis epidemics have been reported in the past years, but usually occurs sporadically with only 2 to 15 cases per million people per year (Farber & Peterkin, 1991; Jacquet *et al.*, 1995). However, 2 500 cases of human listeriosis are still reported annually in the United States and of these cases 500 deaths have been reported (Mead *et al.*, 2006; Montville & Matthews, 2008). Although regulations regarding *L. monocytogenes* contamination in food are implemented and strictly adhered to, the major cause of listeriosis is considered to be the ingestion of contaminated food (Farber & Peterkin, 1991). Six major outbreaks of listeriosis were reported between 1979 and 1999 in North America. The cause was the ingestion of contaminated food sources as

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diverse as commercially prepared coleslaw, lettuce, carrots, pork tongue in jelly, paté, milk that was contaminated after pasteurisation, chocolate milk, soft cheese made from unpasteurised milk and hot dogs (Donnelly, 2001). The listeriosis outbreak due to contaminated hot dogs was attributed to hot dog meat contamination levels of <0.3 cfu g^{-1} (Donnelly, 2001). This further provides evidence for the very low, but still unknown, infectious dose. These relatively recent listeriosis outbreaks due to contaminated food products cause great concern for the food industry and have highlighted the importance and necessity of continued surveillance of pathogens such as *L. monocytogenes* in RTE products (Farber & Peterkin, 1991; Liu *et al*., 2003a; Jacquet *et al*., 1995).

E. Detection methods

To be able to distinguish *L. monocytogenes* from other *Listeria* species, a rapid, specific and sensitive test is essential. The quick and accurate detection of *L. monocytogenes* will lead to the control of the spread of this organism. Two types of detection methods will be discussed, conventional and molecular methods.

Conventional methods

Listeria monocytogenes in food samples often grow in competition with other non-target micro-organisms and are often injured as a result of freezing, heating, drying, irradiation or exposure to chemicals (Bunduki *et al*., 1994). To be able to detect *L. monocytogenes*, a primary and secondary enrichment procedure (half and full strength Fraser broth) is essential to recover the sublethally injured *L. monocytogenes*. After the food samples have been enriched various identification methods, which generally cannot recover the sublethally injured *Listeria*, are used. These methods include cultural and biochemical confirmation, β-hemolysis, the Christie Atkins Munch-Petersen (CAMP) test and more recently chromogenic substrates. In all of these cases, it is important to consider to which extent the identification methods have been validated. Unvalidated methods might provide rapid results, but not necessarily results that are comparable with those of other studies and laboratories. The Association of Analytical Chemists (AOAC) Official Methods provides a variety of methods that has been validated to identify *Listeria* (Gasanov *et al*., 2004) and can be compared between laboratories.

Cultural and biochemical confirmation

The earliest identification methods for *Listeria* species relied on biochemical and phenotypic characteristics and are still widely used. *Listeria* can be cultured on most common bacteriological media. However, attempted isolation of *Listeria* from non-selective media had little success as most food specimens are naturally contaminated with large numbers of other micro-organisms and only contain a small number of *Listeria* spp. (Donnelly, 1999). Inhibition of these indigenous micro-organisms, as well as the resistance of *Listeria* to various antibiotics, have led to the formulation of selective agars. Selective agents such as glycine anhydride, phenylethanol, lithium chloride and antibiotics have been added to the media to enable the isolation of *Listeria* in the presence of Gram-negative bacteria and to inhibit Gram-positive contaminants, such as *Staphylococcus* and *Pseudomonas*. Selective media used for the isolation of *Listeria* include McBride Listeria agar, LPM agar, Oxford agar, MOX agar and PALCAM agar (Donnelly, 1999; Adam & Moss, 2008).

The Henry technique, an oblique illumination technique was developed to facilitate the recognition of *Listeria* colonies on blood-free media. Using the Henry technique, plates are examined under obliquely transmitted white light at an angle of 45° with a binocular scanning microscope. *Listeria* colonies would appear small and round and have a blue-grey to blue-green appearance (Henry, 1933; Adams & Moss, 2008). Some media, such as Oxford agar, incorporates aesculin and ferric ammonium citrate to produce a visible colour change which would eliminate the use of the Henry technique (Adams & Moss, 2008).

Oxford agar is prepared from Columbia agar base, selective agents and aesculin and ferric ammonium citrate, with the latter two as differential agents. The aesculinase reaction is used to differentiate *Listeria* from other bacteria (Curtis *et al.*, 1989). The β-D-glucosidase activity of *Listeria* hydrolyses aesculin, producing a black zone surrounding typical colonies due to the formation of black iron phenolic compounds derived from the aglucon after 48 h of incubation (Greenwood *et al.*, 2005). The selective agents added to Oxford agar, namely lithium chloride, cycloheximide, colistin sulphate, acriflavine, cefotetan and fosfomycin inhibit the growth of other micro-organisms. Unfortunately, *Enterococcus* and *Bacillus* spp. will also grow on these selective plates and also utilise aesculin. Further tests, therefore, are required to conclusively identify colonies of *Listeria*.

These second step tests, used to determine if suspect colonies are *L. monocytogenes*, rely on the unique biochemical and β-hemolysis characteristics of each

of the *Listeria* species (Table 2) as they all exert the same phenotype (morphology) (Sallen *et al*., 1996; Montville & Matthews, 2008). These tests include acid production from xylose, rhamnose, mannitol and alpha-methyl-D-mannoside, β-hemolysis and the Christie Atkins Munch-Petersen (CAMP) test. These tests can take up to a week to obtain results and as commercial identification kits can produce the same results in a shorter period of time, they are widely used. These kits, such as the API *Listeria* (bio-Merieux, France) and Micro-ID (Remel, USA) have been validated and are incorporated into standard methodology (Hitchins, 2001).

β - hemolysis

The addition of blood to solid media can also be used to differentiate between *Listeria* species. Three *Listeria* species, *L. monocytogenes, L. ivanovii* and *L. seeligeri* produce hemolysins resulting in a zone of β-hemolysis on blood agar plates. In contrast *L. grayi*, *L. innocua* and *L. welshimeri* do not produce hemolysins and are, therefore, non-hemolytic. The ability to lyse red blood cells differentiates the potentially pathogenic *Listeria* species from non-pathogenic *Listeria* species. The zones of hemolysis produced by *L. ivanovii* are prominent and comprise two distinctive zones, a clear zone and a partial zone of lysis. The clear zone is due to the action of ivanolysin O (58 kDa) and is nearest to the colony, while the partial hemolysis zone is caused by sphingomyelinase (27 kDa) and lecithinase. The hemolysis zone produced by *L. monocytogenes* and *L. seeligeri* are more modest and show narrow, slight clearing zones which are not always visible (Montville & Matthews, 2008). Hemolysis is an important characteristic for differentiation between the species, but is difficult to detect. A study by Higgins and Robinson (1993) noted that inaccurate readings of the hemolysis resulted in a large percentage of errors in the identification of *L. seeligeri* and *L. ivanovii.* Further tests are, therefore, needed to correctly identify the different species.

The Christie Atkins Munch-Petersen (CAMP) test

The CAMP test (Groves & Welshimer, 1977) is a useful diagnostic test used to differentiate between the haemolytic *Listeria* species *L. ivanovii*, *L. monocytogenes* and *L. seeligeri*. A blood agar plate is used and a β-hemolysin-producing *Staphylococcus aureus* strain and *Rhodococcus equi* is streaked parallel to each other. Suspect colonies are streaked out at right angles between the two streaks, without touching

Table 2 Biochemical properties of *Listeria* species (Adapted from Swaminathan *et al*., 1995; Bhunia, 2008).

Characteristics	***L. monocytogenes***	***L. innocua***	***L. ivanovii***	***L. welshimeri***	***L. seeligeri***	***L. grayi***
β-hemolysin	+	-	+	-	+	-
CAMP (*S. aureus*)	+	-	-	-	+	-
CAMP (*R. equi*)	-	-	+	-	-	-
L-Rhamnose	+	v	-	-	v	-
D-Xylose	-	-	+	+	+	-
α-Methyl-D-mannoside	+	+	-	+	-	+
Mannitol	-	-	-	-	-	+
Cytotoxicity	+	-	+	-	±	-
Invasion assay	+	-	+	-	-	-
Mouse virulence	+	-	+	-	-	-

CAMP - Christie, Atkinson, Munch-Peterson hemolysis assay; + positive; - negative; v variable

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(Vazquez-Boland *et al*., 1990). A positive result is indicated by an enhanced zone of hemolysis at the intersection of the test strains with either *S. aureus* or *R. equi* (Rocourt, 1999). Hemolysis by *L. monocytogenes* is enhanced in the vicinity of *S. aureus*, due to the production of a 58 kDa β-hemolysin, listerolysin O, which acts in synergy with the hemolysin produced by *S. aureus.* This enhances the hemolysis on the blood agar plate (Adams & Moss, 2008). Hemolysis by *L. ivanovii* is enhanced in the vicinity of the *R. equi* streak and produces a clear zone of hemolysis resembling an arrowhead or shovel-like shape (Bhunia, 2008). This test was examined for its speciation applicability and was shown to sometimes incorrectly differentiate between *L. monocytogenes* and *L. ivanovii* (Vazquez-Boland *et al*., 1990). A study by Higgins and Robinson (1993) also noted that a considerable percentage of errors were caused by inaccurate reading of the tests when *L. ivanovii* and *L. seeligeri* were investigated.

Chromogenic substrates

The recent commercial availability of chromogenic media allows direct identification of colonies by their characteristic colour. Chromogenic substrates are incorporated into the plating media and provide rapid identification of the bacteria based on their bacterial enzymes. These media have many advantages over other tests in being simple, easy to interpret, cost effective, highly sensitive and specific and allowing a large sample throughput. The activity of the enzyme phosphatidylinositol-specific phospholipase C (PI-PLC) is measured by the opaque white halo colour reaction produced by the hydrolysis of phosphotidylinositol or lethicin for *L. monocytogenes* and *L. ivanovii* identification (Coffey *et al*., 1996). A commercially available chromogenic agar, Rapid'*L. mono* agar (Bio-Rad, USA) is used for the detection of *L. monocytogenes*, producing blue colonies for PI-PLC-positive bacteria. Although *L. ivanovii* also produce blue colonies, xylose fermentation is used to further differentiate between *L. monocytogenes* and *L. ivanovii. Listeria monocytogenes* retains the red media background, being unable to ferment xylose and *L. ivanovii*, being able to ferment xylose produces a yellow media background (Sacchetti *et al*., 2003).

Molecular methods

One of the biggest difficulties encountered during detection of *L. monocytogenes* is the low numbers of the bacteria found in contaminated food samples (Hoffman & Weidmann, 2001). Molecular methods have proven to be useful in detecting low numbers of *L. monocytogenes*, due to the fact that low concentrations of DNA can be

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specifically detected or amplified (Churchill *et al*., 2006). Molecular methods detect differences at the DNA level and can differentiate between micro-organisms at the genus, species and even subspecies level (Liu *et al*., 2003a). According to the dictionary, type'-ing (noun) is a number of things or persons sharing a particular characteristic, or set of characteristics, that causes them to be regarded as a group, more or less precisely defined or designated, class, category. In biology it is defined as a genus or species that most nearly exemplifies the essential characteristics of a higher group, or the one or more specimens on which the description and naming of a species is based. Subtype'-ing, according to the dictionary is a group forming a type within a larger type. Typing, therefore, refers to the typing of species in a genus and subtyping refers to the typing of different strains within a species (Anon., 2010b).

According to the study by Klæboe *et al*. (2005) it was identified that molecular subtyping is crucial in the control of *L. monocytogenes* in processing plants. This information is needed to identify niches and transmission routes which will enable the implementation and design of control strategies at various stages of production in order to reduce the occurrence of *L. monocytogenes*, as well as identifying whether the strains present are virulent or non-virulent (Roche *et al*., 2003; Liu *et al*., 2003a; Klæboe *et al*., 2005).

For the subtyping of *L. monocytogenes*, there are two approaches that are commonly used, namely phenotypic and genotypic subtyping. Subtyping by phenotypic methods includes phage typing, serotyping, multilocus enzyme electrophoresis (MLEE) and esterase typing. The molecular subtyping methods includes DNA sequencing-based subtyping techniques [for example multilocus sequence typing (MLST)], ribotyping, PCR-based subtyping techniques [for example random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), PCR-restriction fragment length polymorphism (PCR-RFLP) and repetitive element PCR (REP-PCR)] and pulsed-field gel electrophoresis (PFGE). The molecular subtyping methods are reproducible, discriminatory and highly sensitive, while the phenotypic methods have low discrimination and reproducibility. This can be attributed to the fact that phenotypic characteristics are not only determined by the genotype, but also the environmental conditions (Singleton & Sainsbury, 1978). The six *Listeria* species have close morphological and biochemical resemblances, therefore, a test that uses the unique genetic characteristics of the organism is needed (Liu, 2006).

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F. Molecular subtyping techniques

DNA sequencing-based subtyping

Multilocus sequence typing (MLST)

The MLST technique was developed by Maiden *et al*. (1998) and it based on the principles of MLEE. This technique targets multiple genetic loci among different strains within a species that have changed slowly over time. MLST uses automated DNA sequencing to characterise the alleles present at different housekeeping genes to subsequently define an allelic profile or sequence type for each isolate (Salcedo *et al*., 2003). The advantage of this technique is that the results, the sequence data, are unambiguous. These results can be placed in a central database and it can be accessed through a web server, thus enabling exchange of molecular typing data for global epidemiology (Maiden *et al*., 1998).

Kalpana *et al*. (2004) used MLST for the subtyping of *L. monocytogenes* among five genetic loci which encodes for four virulence genes. These included the *hly*A, *prf*A, *inl*A and *act*A genes, all part of the LIPI-1 virulence cluster, except for *inl*A. A study by Salcedo *et al*. (2003) analysed nine housekeeping genes in a set of 62 *L. monocytogenes* strains from different sources and regions in Spain using MLST. These strains were previously characterised by PFGE and the sequence analysis of the MLST were generally consistent with those of PFGE, providing similar clustering of the strains, except for one isolate which showed a high number of fragment differences.

Parisi *et al*. (2010) used both MLST (analysing seven housekeeping genes) and AFLP to study 103 *L. monocytogenes* isolates from food and environmental sources in Italy. A total of 66 MLST sequence types and 62 AFLP types were identified. Both methods exhibited comparable discriminatory powers, 0.972 and 0.976 for MLST and AFLP, respectively, with both being higher than that of serotyping (0.739). Parisi *et al*. (2010) concluded that both methods are suitable tools for subtyping *L. monocytogenes,* but that MLST had the advantage over AFLP of producing unambiguous sequence data which permits inter-laboratory comparisons.

A study by Jiang *et al*. (2008) analysed 19 *L. monocytogenes* food-related isolates from eastern China using PFGE and MLST (analysing three virulence genes and four housekeeping genes). They concluded that virulence based MLST had a far better discriminatory power than the MLST targeting the housekeeping genes (0.990 vs 0.895) and that it was similar to PFGE (0.976).

Although MLST is user-friendly, not as expensive as ribotyping nor as laborious as PFGE (Kalpana et al., 2004), it is still more expensive and time-consuming than

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AFLP. Unfortunately, despite the standardisation of the method, there is no common method or scheme specific for *L. monocytogenes* and, therefore, this method may become the gold standard for *L. monocytogenes* subtyping in the future (Parisi *et al*., 2010).

Ribotyping

Many bacterial pathogens are subtyped using ribotyping as it is a powerful and discriminatory method that has become very popular (Nair *et al*., 1999). The method is similar to RFLP in that it also digests DNA with restriction endonucleases to create gel images with unique band patterns (Churchill *et al*., 2006).

The method involves the extraction of DNA, followed by digestion with restriction endonucleases. After digestion, Southern blotting is performed and the transferred DNA is then hybridised with labelled rRNA gene probes (Nair *et al*., 1999). Only DNA fragments with homologous sequences will be detected by the probe on the blot (Ryser *et al*., 1996; Weidmann *et al*., 1997). Computer programs (Riboprinter® Microbial Characterisation System (DuPont Qualicon™ Inc., USA)) are then used to analyse the resultant band patterns (Nair *et al*., 1999). This method relies on the relatively conserved ribosome encoding genes, which with mutations over time, allows the determination of lineages with the different band patterns obtained (Churchill *et al*., 2006).

A study by Weidmann *et al*. (1997) demonstrates the subtyping of *L. monocytogenes* isolates by ribotyping. They digested the DNA using *Eco*RI and used the *E. coli rrnB* rRNA operon probe. The 133 strains investigated were divided into 23 different groups based on the band patterns obtained. This information was then combined with analysis of polymorphism in the virulence genes *act*A, *hly*A and *inl*A and showed that the strains could be divided into three distinctive lineages. Only the *hly*A gene divided the lineages into groups of strains that correlated to listeriosis outbreaks (Weidmann *et al*., 1997).

Meloni *et al.* (2009) investigated the prevalence of *L. monocytogenes* in RTE foods of animal and vegetable origin in Sardinia and used automated *Eco*RI ribotyping to analyse the potential virulence of the strains. *Eco*RI ribotyping differentiated the 42 *L. monocytogenes* isolates into 16 distinct ribotypes with a discrimination index of 0.911. Cluster analysis of the ribotypes indicated that 65 % of the strains belonged to lineage II (serotypes 1/2a and 1/2c) which are known to be associated with sporadic human listeriosis outbreaks. The remaining 35 % of the isolates were allocated to lineage I

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(serotypes 1/2b, 3b and 4b) which have also been associated with listeriosis outbreaks. Meloni *et al.* (2009) concluded that *Eco*RI ribotyping is a rapid and reliable method for the subtyping of *L. monocytogenes* providing useful data for epidemiologic surveys.

Manfreda *et al*. (2005) used ribotyping for the subtyping of *L. monocytogenes* isolates (n=1 656) from contaminated Gongonzala cheeses. The data revealed that 70 % of the isolates had the same ribotype. It also showed that 90 % of the isolates were associated with type II pathogenicity lineage (Manfreda *et al*., 2005). Mereghetti *et al*. (2002) used ribotyping and random multiprimer DNA analysis to analyse the type II lineage. They found that lineage II is more heterogeneous than lineage I and that it most likely diverged earlier from the common ancestor than lineage I.

A study by Lukinmaa *et al*. (2004) demonstrated the use of automated ribotyping, PFGE and serotyping for *L. monocytogenes* isolates collected from human blood and the food industry in Finland during 1997 – 1999. The isolates were divided into 23 *Eco*RI ribotypes, 54 *Asc*I PFGE types and six serotypes. Two ribotypes included isolates of two serotypes, whereas all the PFGE types were serotype-specific. One of these two ribotypes included a serotype 3a isolate that was involved in a listeriosis outbreak in Finland in 1999, as well as a serotype 1/2a food industry isolate. Lukinmaa *et al*. (2004) concluded that ribotyping would not have been sufficient to define the outbreak caused by serotype 3a isolates in Finland and, therefore, human and food isolates with the same ribotypes should be further investigated by PFGE.

PCR-based subtyping techniques

PCR has many advantages including high throughput processing, a level of automation, and a relatively short reaction time. It also shows an increase in sensitivity over culturing methods. The inability of some cells to grow in selective media due to low numbers is also excluded as PCR amplifies the specific genetic signals from as little as only a few cells (Shearer *et al*., 2001; Aznar & Alarcón, 2002).

Arbitrarily primed PCR (AP-PCR) and randomly amplified polymorphic DNA (RAPD)

Both RAPD and AP-PCR uses low-stringency PCR amplification to generate anonymous DNA fragments that are strain-specific. The primers used are single, short (approximately 10 bases long for AP-PCR and 10 – 15 bases for RAPD) and of arbitrary sequence. These primers are used at relatively low temperatures (around 36 °C) during PCR, thus effectively lowering the stringency of the primer-annealing temperature. This

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in turn allowing the annealing of a random primer that shows a perfect match of two or three nucleotides between the template strand and the 3' end of the primer. This three-nucleotide sequence can presumptively be found in each 64 nucleotide sequences (4^3 permutations). When two such events (annealing and priming) occur within a certain distance from one another, the sequence that lies between these two sites can be amplified (Farber & Addison, 1994; O'Donoghue *et al*., 1995). A comparison between strains or isolates can only be made if the same primer is used for all the test samples. Otherwise, the fragments yielded would be of no importance as the amount and sizes of the fragments would conflict because of the different primers used. The test samples also have to be pure cultures to avoid contamination and subsequent deceiving or misleading results (Lawrence & Gilmour, 1995).

This technique has been used to trace the source of *L. monocytogenes* contamination in both poultry (Lawrence & Gilmour, 1995) and vegetable (Aguado *et al*., 2004) processing plants. In the year-long study by Lawrence & Gilmour (1995), samples were taken throughout the year, cultured, and RAPD analysis was performed on the isolates. The results indicated that there were two strains of *L. monocytogenes* that remained in the processing plant throughout the year. These two strains were persistent and was likely responsible for cross-contamination of the preparation areas. They were able to determine the source of the contamination as the incoming birds and also demonstrated that there are transitory strains that were isolated which probably came from a variety of contamination sources.

In a study by Ertaş and Şeker (2005) the presence and genetic variation of *L. monocytogenes* in fresh fish caught from the Keban Dam Lake, Turkey, was investigated. Of the 150 fish (*Capoeta capoeta umbla*) caught, only 10 fish were contaminated with *L. monocytogenes*. The genetic variation of these 10 isolates were analysed by RAPD. In the analysis, two distinguishable and reproducible band profiles were obtained. The different band profiles indicate the possibility that the *L. monocytogenes* are from different sources. However, more discriminative typing techniques should be used to reveal the relationship between the fish isolates (Ertaş & Şeker, 2005).

Franciosa *et al*. (2001) investigated 32 *L. monocytogenes* strains involved in invasive and non-invasive listeriosis outbreaks in Italy using PCR-ribotyping, AP-PCR and infrequent-restriction-site PCR (IRS-PCR). The discriminatory ability of the three techniques was evaluated and was found to be 0.714, 0.690 and 0.919 for PCR-ribotyping, AP-PCR and IRS-PCR, respectively. IRS-PCR identified three clusters

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among the strains of the invasive listeriosis outbreak compared to only two clusters by PCR-ribotyping and AP-PCR each. Within each of the two non-invasive listeriosis outbreaks, the patterns obtained were practically identical, confirmed by all three techniques. Only IRS-PCR could clearly discriminate between the strains of the non-invasive and the invasive listeriosis outbreaks.

RAPD and AP-PCR are faster and more economical than other subtyping techniques and, therefore, particularly suitable when testing less than 50 strains (Farber & Addison, 1994; O'Donoghue *et al*., 1995; Louie *et al*., 1996). It can also be used effectively for microbial source tracking and the results obtained can give an indication of contamination sites within a food processing plant (Lawrence & Gilmour, 1995; Churchill *et al*., 2006). When at least two independent primers are used for AP-PCR, the discriminatory power is comparable to that of PFGE (Louie *et al*., 1996). The disadvantage, however, is the inconsistency in the discriminatory ability (Farber & Addison, 1994; O'Donoghue *et al*., 1995). Franciosa *et al*. (2001) reported that AP-PCR gave less discriminatory results than ribotyping for the subtyping of *L. monocytogenes* isolates involved in listeriosis outbreaks (Table 3).

Amplified fragment length polymorphism (AFLP)

AFLP is a fingerprinting technique based on the amplification of genomic restriction fragments to create a restriction pattern of the amplified bands (Aarts *et al*., 1999). DNA is digested with two restriction enzymes, one that cuts infrequently and another that cuts more frequently (Blears *et al*., 1998). After the DNA is digested, it is ligated with double-stranded adapter oligonucleotides (Aarts *et al*., 1999). It is then amplified by PCR using adapter-specific primers (Keto-Timonen *et al*., 2003). This will result in the amplification of only those fragments that have the same complementary sequence between the adapter and the restriction site and will thus result in amplification of 1/16 of the bands (Aarts *et al*., 1999). After PCR, the products are separated by polyacrylamide gel electrophoresis, to yield highly informative, polymorphic patterns (Ripabelli *et al*., 2000; Guerra *et al*., 2002; Keto-Timonen *et al*., 2003).

AFLP is a sensitive, reproducible and highly discriminatory method that can be used to discriminate between species of the genus *Listeria*, as well as between different *L. monocytogenes* strains (Ripabelli *et al*., 2000; Guerra *et al*., 2002; Keto-Timonen *et al*., 2003). The disadvantage of AFLP is the ligation of adapters, which is not only a time-consuming step, but also adds ambiguity to the method (Liu, 2006).

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Vogel *et al*. (2004) used AFLP, RAPD, PFGE, serotyping and ribotyping to classify 96 different strains of *L. monocytogenes*. Results from AFLP indicated that the strains could be divided into 45 groups, with similar results obtained by RAPD and PFGE. Both ribotyping and serotyping was not as discriminatory, grouping the strains into only 16 and 2 groups, respectively (Vogel *et al*., 2004). A study by Autio *et al*. (2003) also reported that AFLP results obtained were in accordance with the PFGE results obtained, having a high discriminatory power and reproducibility.

Parisi *et al*. (2010) used both AFLP and MLST to study 103 *L. monocytogenes* isolates from food and environmental sources in Italy. Both techniques had similar results (62 AFLP types and 66 MLST sequence types identified) and were more discriminatory than serotyping. Parisi *et al*. (2010) concluded that both methods are suitable tools for subtyping *L. monocytogenes,* but that AFLP had the advantage of being less expensive, highly advancing and easily identify different *Listeria* species.

PCR-RFLP

This method uses restriction endonucleases to cut DNA into fragments of different lengths to obtain different band patterns. These band patterns can then be used to determine differences in the genetic profiles of the organism. Even closely related individuals have mutations at the site of restriction in sufficient frequency to form different lineages (Smith & Nelson, 1999). When genomic DNA is digested using restriction endonucleases, separated using electrophoresis and analysed, different electrophoretic patterns of DNA bands are visible, with a very high number of bands (Table 3). Using genomic DNA would result in a gel containing an abundant amount of DNA bands without adequate distinction between them. This makes the interpretation of the results very difficult (Churchill *et al*., 2006). It is, therefore, preferred to use an individual gene to minimise the amount of DNA bands visible on the gel, which will result in an easier interpretation of the different patterns. The method is, however, time consuming due to the need for using pure cultures (Paillard *et al*., 2003).

Weidmann *et al*. (1997) used the *hly*A, *act*A and *inl*A genes of *L. monocytogenes* for RFLP analysis. According to the results, *L. monocytogenes* could be divided into three lineages. They also found that lineage I was responsible for all epidemic outbreaks. The relationship of different strains in terms of pathogenic potential could, therefore, be determined (Weidmann *et al*., 1997).

Rousseaux *et al*. (2004) used a PCR-RFLP method to screen a large amount of *L. monocytogenes* strains for impaired adhesion to epithelial cells due to the expression

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Table 3 Comparison of pathogen subtyping and verification methods (Adapted from Savelkoul *et al*., 1999; Gurtler & Mayall, 2001).

Methods	Advantages	Disadvantages	Amount of DNA required
AFLP	PCR to amplify a few genes and simplify analysis of results. Differences between bands on a gel comparable. Fewer bands to compare than with RFLP. Can compare between labs.	Requires pure cultures of pathogen. Cannot compare organisms at the genus or family level.	10 – 100 ng
PFGE	PulseNet database available to coordinate and compare results so outbreaks can be tracked. Differences between bands on a gel comparable. Results are comparable among labs.	Time consuming – 3 days for results. Requires pure cultures of pathogen. Patterns may change after intestinal passage; differences in patterns may not indicate actual strain differences. Cannot compare organisms at the genus or family level.	Measured by turbidity of culture – lysis and DNA digestion performed in agarose plugs
RAPD	Arbitrary primers that are not gene specific. No knowledge of genome sequence	Requires pure cultures of pathogen. Is not very reproducible between gels or labs. Cannot compare organisms at the genus or family level.	10 ng
RFLP	Simplicity, can be done with restriction endonucleases for serotype level discrimination or combined with PCR for lineage comparisons.	Requires pure cultures of pathogen. Many bands to compare, thus confusing, may be hard to see differences. Cannot compare organisms at the genus or family level. Partial digestion and faint bands may be a problem.	3 – 5 µg
Ribotyping	Similar to RFLP, but visualises only bands corresponding to the *rrn* portion of ribosomes so fewer bands to compare. Can compare organisms at the genus and family level.	Requires pure cultures of pathogen.	1 µg

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of truncated InlA. Two endonucleases, *Alu*I and *Tsp*509I, were used and generated five composite profiles for the 37 strains analysed. The method allowed Rousseaux *et al.* (2004) to identify 10 new strains expressing truncated internalin.

REP-PCR

Listeria monocytogenes, like other prokaryotic organisms, contains a genome with repetitive sequence elements, which are randomly dispersed throughout the genome. They include enterobacterial repetitive intergenic consensus sequences (ERICs) which contains 124 – 147 base pairs (bp) and have a highly conserved central inverted repeat and repetitive extragenic palindromes (REPs) which contain 35 – 40 bp and which also have an inverted repeat. These ERIC and REP sequences are both ideal primer binding sites for PCR amplification and could be used for both species and strain discrimination.

A study by Jersek *et al.* (1999) used REP-PCR on *L. monocytogenes* strains and divided them into four clusters. These clusters matched the origin of isolation and each cluster had several subtypes. A study by Chou and Wang (2006) investigated the genetic relationship between *L. monocytogenes* isolates from seafood and human origin using PFGE and REP-PCR. A total of 128 isolates were analysed, 36 from catfish, 35 from non-catfish seafood and 57 from humans. Using PFGE (both *Apa*I-digestion and *Asc*I-digestion) and REP-PCR revealed 97, 87 and 94 subtypes, respectively. Among all of the subtypes, only two *Apa*I-digested subtypes, one *Asc*I-digested subtype and three REP-PCR subtypes were shared by catfish or non-catfish seafood with human isolates. Chou and Wang (2006) concluded that REP-PCR has a similar discriminatory ability as PFGE. REP-PCR represents a useful alternative for subtyping *L. monocytogenes* as it is rapid, less expensive and has a similar level of discrimination to ribotyping and PFGE methods (Chou & Wang, 2006; Liu, 2006).

G. Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) is based on the RFLP method (Liu, 2006) and allows the separation of large DNA fragments from 10 – 2000 kilo base pairs (kbp) (Finney, 2000). The size limit of a normal agarose gel is around 20 to 40 kilo base pairs (kb). If larger fragments are separated, they would migrate at the same rate and be visual on the gel as a single band (Churchill *et al.*, 2006).

Pulsed-field gel electrophoresis uses this characteristic as a basis for further separation. Large DNA fragments takes longer than smaller fragments to change into

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their elongated shapes for movement. By changing the direction of the electric field, it allows the smaller fragments to alter their shape faster and thus commence migration at the limiting mobility rate. By changing the angles and times of the electrophoretic field, it allows the resolution of larger DNA fragments (Moore & Datta, 1994; Finney, 2000).

Pulsed-field gel electrophoresis is divided into three steps, namely the preparation of agarose plugs with unbroken genomic DNA incorporated into it, the digestion of these plugs with infrequently cutting restriction nucleases to produce large digested fragments (Yde & Genicot, 2004) and the electrophoresis of these fragments using PFGE, allowing separation of fragments ranging in size from 10 000 base pairs (10 kb) to 2 million base pairs (2 Mb) (Finney, 2000; Smith *et al*., 1987). The preparation of agarose plugs minimises the shearing and loss of DNA, which are commonly associated with the liquid phase phenol extraction method. The DNA is, therefore, more intact and can be stored for longer periods. The preparation of these plugs also decreases the risk of exposure of virulent human pathogens to laboratory workers (Nair *et al*., 1999).

Several authors have applied this technique successfully for the epidemiological investigations of listeriosis (Carrière *et al*., 1991; Brosch *et al*., 1991, 1994; Buchrieser *et al*., 1993; Nguyen *et al*., 1994; Jacquet *et al*., 1995; Proctor *et al*., 1995; Louie *et al*., 1996). Subtypes within isolates which is indistinguishable by other subtyping methods are identifiable by PFGE (Buchrieser *et al*., 1993; Brosch *et al*., 1991, 1994; Jacquet *et al*., 1995; Louie *et al*., 1996).

Moore and Datta (1994) used PFGE on *L. monocytogenes* isolated from two listeriosis outbreaks in the United States. They digested the DNA with *Sma*I and analysed the band patterns. They found that the two listeriosis outbreaks were not as closely related as previous data from serotyping suggested. In terms of banding patterns, the isolates of each individual outbreak was more closely related to one another, than between outbreaks. However, the serotyping suggested that the two outbreaks might be clonal since they had the same serotype (Moore & Datta, 1994).

Serotyping, ribotyping, PFGE and AFLP was used and compared in a study by Corcoran *et al*. (2006). Fourty-six *L. monocytogenes* isolates, 26 from smoked salmon products and 20 from various other food sources were analysed. Serotyping grouped the isolates into four serotypes, ribotyping into 12 distinct ribotypes, PFGE into 17 distinct DNA fragment profiles and AFLP into 12 distinct AFLP profiles. However, serotyping had four isolates that was non-typeable and AFLP had three isolates that was also non-typeable. Corcoran *et al*. (2006) concluded that although PFGE requires

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specialised equipment and human resources, it is an excellent and reproducible method for subtyping *L. monocytogenes*.

Vogel *et al*. (2004) compared PFGE typing to serotyping for the subtyping of *L. monocytogenes*. The restriction endonuclease that was used for the restriction step in PFGE was *Apa*I. They found that PFGE was more discriminatory and that it sorted the strains into more groups than the serotyping. Of the 96 *L. monocytogenes* strains investigated, the PFGE results indicated that 39 groups could be identified while serotyping results only divided the strains into two groups (Vogel *et al*., 2004).

Aarnisalo *et al*. (2003) compared PFGE to ribotyping, and found that PFGE was more discriminating than ribotyping. Of the 486 *L. monocytogenes* isolates from 17 processing plants in Finland, 46 types were identified using PFGE, while only 23 ribotypes could be identified using ribotyping (Aarnisalo *et al*., 2003).

PFGE is currently regarded as the gold standard method for the molecular subtyping of foodborne bacteria, including *L. monocytogenes,* due to the high discrimination of this technique, reproducibility and the fact that the method is standardised (Gerner-Smidt *et al*., 2006). The advantage of PFGE being highly reproducible permits the construction of a database based on the different band patterns obtained. This database can then be used for surveillance and also aid in determining the possible vehicle of transmission or infection in listeriosis outbreaks or contaminations in processing plants (Lyytikainen *et al*., 2006).

An electronic database known as PulseNet was initiated by the US Centre for Disease Control (CDC) in collaboration with many public laboratories. The database catalogs the PFGE patterns of four organisms: *E. coli* O157:H7, non-typhoidal *Salmonella* serotypes, *Shigella* and *L. monocytogenes* (Swaminthan *et al*., 2001). As from 2002, the FDA, US Department of Agriculture (USDA), 46 state labs, 2 public labs and several Canadian labs have contributed to the database. This has led to the earlier detection and better tracking of possible common source outbreaks (Swaminthan *et al*., 2001; Kathariou, 2002). While this method has the advantage of providing a lot of information regarding strains and their differences, it is time consuming, requiring at least three days to obtain results (Table 3) (Finney, 2000).

H. Conclusion

The contamination of RTE and refrigerated food products with *L. monocytogenes* is a serious problem. Ready-to-eat products do not receive harsh treatments to ensure

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microbiological safety and subsequently, when stored under certain conditions, may favour the growth and multiplication of the pathogenic micro-organism, *L. monocytogenes* (WHO/FAO, 2004). It is, therefore, no surprise that manufacturers of RTE foods have taken notice of these species as an increase in the consumption of RTE foods and meals served outside the home (Kennedy & Wall, 2007) have also led to the recognition of *L. monocytogenes* as an important opportunistic human foodborne pathogen (Liu, 2006). Not only does it cause severe economic losses for the industry, but also poses as a health risk to humans.

Several methods are available to detect and identify *L. monocytogenes* contamination. The conventional methods, which are culture-dependent, are time-consuming and may result in inaccurate results. Molecular methods, being culture-independent, relies on the genetic content of the organism and therefore result in more accurate and reproducible results.

A technique that can differentiate between the different strains of *L. monocytogenes* is essential to determine a source of contamination. Multilocus sequence typing, ribotyping, AP-PCR, RAPD, AFLP, PCR-RFLP, REP-PCR and PFGE techniques have all been used for the fingerprinting or subtyping of *L. monocytogenes*. PFGE is an excellent technique to use as it uses the whole genome and not a single gene to differentiate between the strains and could subtype isolates that were indistinguishable by other subtyping methods.

I. References

Aarnisalo, K., Autio, T., Sjöberg, A.-M., Lundén, J., Korkeala, H. &Suihko, M.-L. (2003). Typing of *Listeria monocytogenes* isolates originating from the food processing industry with automated ribotyping and pulsed-field gel electrophoresis. *Journal of Food Protection*, **66**, 249–255.

Aarts, H.J.M., Hakemulder, L.E. & Van Hoef, A.M.A. (1999). Genomic typing of *Listeria monocytogenes* strains by automated laser fluorescence analysis of amplified fragment length polymorphism fingerprint patterns. *International Journal of Food Microbiology*, **49**, 95–102.

Adams, M.R. & Moss, M.O. (2008). Bacterial agents of foodborne illness. In: *Food Microbiology*, 3rd ed. Pp 224–231. Cambridge: RSC Publishing.

Aguado, V., Vitas, A.I. & García-Jalón, I. (2004). Characterization of *Listeria monocytogenes* and *Listeria innocua* from a vegetable processing plant by RAPD and REA. *International Journal of Food Microbiology*, **90**, 341–347.

Stellenbosch University http://scholar.sun.ac.za

Altekruse, S.F., Cohen, M.L. & Swerdlow, D.L. (1997). Emerging foodborne diseases. *Emerging Infectious Diseases*, **3**, 285–293.

Anonymous (2005). Commision regulation No. 2073/2005 on microbiological criteria for food stuffs. *Official Journal of the European Union*, L388, Pp 1–26.

Anonymous (2009). *Abstract of Agricultural Statistics 2009*. Pretoria: Department of Agriculture.

Anonymous (2010a). Industry statistics. [WWW document]. URL http://www.subtrop.net/home.html. 15 February 2010.

Anonymous (2010b). [WWW document]. URL http://www.dictionary.com. 15 February 2010.

Aureli, P., Fiorucci, G.C., Caroli, D., Marchiaro, G., Novara, O., Leone, L. & Salmaso, S. (2000). An outbreak of febrile gastroenteritis associated with corn contaminated by *Listeria monocytogenes*. *The New England Journal of Medicine* **342**, 1236–1241.

Autio, T., Keto-Timonen, R., Lundén, J., Björkroth, J. & Korkeala, H. (2003). Characterisation of persistent and sporadic *Listeria monocytogenes* strains by pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP). *Systematic and Applied Microbiol*ogy, **26**, 539–545.

Aznar, R. & Alarcón, B. (2002). On the specificity of PCR detection of *Listeria monocytogenes* in food: a comparison of published primers. *Systematic and Applied Microbiol*ogy, **25**, 109–119.

Aznar, R. & Elizaquível, P. (2008). Reliability of *Listeria monocytogenes* identification by specific PCR assessed by phenotypic and genotypic techniques. *Food Analytical Methods*, **1**, 243–251.

Batt, C.A. (1997). Molecular diagnostics for dairy-borne pathogens. *Journal of Dairy Science*, **80**, 220–229.

Bhunia, A.K. (2008). *Listeria monocytogenes*. In: *Foodborne microbial pathogens*. Pp 165–182. New York: Springer Science+Business Media.

Bille, J., Catimel, B., Bannerman, E., Jacquet, C., Yersin, M.N., Caniaux, I., Monget, D. & Rocourt. J. (1992). API *Listeria*, a new and promising one-day system to identify *Listeria* isolates. *Applied and Environmental Microbiology*, **58**, 1857–1860.

Blackman, I.C. & Frank, J.F. (1996). Growth of *Listeria monocytogenes* as a biofilm on various food-processing surfaces. *Journal of Food Protection*, **59**, 827–831.

Stellenbosch University http://scholar.sun.ac.za

Blais, B.W., Turner, G., Sooknanan, R. & Malek, L.T. (1997). A nucleic acid sequence-based amplification system for detection of *Listeria monocytogenes hlyA* sequences. *Applied and Environmental Microbiology*, **63**, 310–313.

Blears, M.J., de Grandis, S.A., Lee, H. & Trevors, J.T. (1998). Amplified fragment length polymorphism (AFLP): a review of the procedure and its applications. *Journal of Industrial Microbiology*, **21**, 99–114.

Borucki, M.K., Peppin, J.D., White, D., Loge, F. & Call, D.R. (2003). Variation in biofilm formation among strains of *Listeria monocytogenes*. *Applied and Environmental Microbiology*, **69**, 7336–7342.

Briandet, R., Leriche, V., Carpentier, B. & Bellon-Fontaine, M. (1999). Effects of the growth procedure on the surface hydrophobicity of *Listeria monocytogenes* cells and their adhesion to stainless steel. *Journal of Food Protection*, **62**, 994–998.

Brosch, R., Buchrieser, C. & Rocourt, J. (1991). Subtyping of *Listeria monocytogenes* serovar 4b by use of low-frequency-cleavage restriction endonucleases and pulsed-field gel electrophoresis. *Research in Microbiology*, **142**, 667–675.

Brosch, R., Chen, J. & Luchansky, J.B. (1994). Pulsed-field fingerprinting of *Listeriae*: identification of genomic divisions for *Listeria monocytogenes* and their correlation with serovars. *Applied and Environmental Microbiology*, **60**, 2584–2592.

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

Buchrieser, C., Brosch, R., Catimel, B. & Rocourt, J. (1993). Pulsed-field gel electrophoresis applied for comparing *Listeria monocytogenes* strains involved in outbreaks. *Canadian Journal of Microbiology*, **39**, 395–401.

Bunduki, M.M., Flanders, K.J. & Donnelly, C.W. (1994). Metabolic and structural sites of damage in heat- and sanitizer-injured populations of *Listeria monocytogenes*. *Journal of Food Protection,* **58**, 410–415.

Carrie`re, C., Allardet-Servent, A., Bourg, G., Audurier, A. & Ramuz, M. (1991). DNA polymorphism in strains of *Listeria monocytogenes*. *Journal of Clinical Microbiology*, **29**, 1351–1355.

Chavant, P., Martinie, B., Meylheuc, T., Bellon-Fontaine, M.-N. & Hebraud, M. (2002). *Listeria monocytogenes* LO28: surface physicochemical properties and ability to form biofilms at different temperatures and growth phases. *Applied and Environmental Microbiology,* **68**, 728–737.

Stellenbosch University http://scholar.sun.ac.za

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

Chou, C.-H. & Wang, C. (2006). Genetic relatedness between *Listeria monocytogenes* isolates from seafood and humans using PFGE and REP-PCR. *International Journal of Food Microbiology*, **110**, 135–148.

Churchill, R.L.T., Lee, H. & Hall, J.C. (2006). Detection of *Listeria monocytogenes* and the toxin listeriolysin O in food. *Journal of Microbiological Methods*, **64**, 141–170.

Coffey, A., Rombouts, F.M. & Abee, T. (1996). Influence of environmental parameters on phosphatidylcholine phospholipase C production in *Listeria monocytogenes*: a convenient method to differentiate *L. monocytogenes* from other *Listeria* species. *Applied and Environmental Microbiology*, **62**, 1252–1256.

Cole, M.B., Jones, M.V. & Holyoak, C. (1990). The effect of pH, salt concentration and temperature on the survival and growth of *Listeria monocytogenes*. *Journal of Applied Bacteriology*, **69**, 63–72.

Curtis, G.D.W., Mitchell, R.G., King, A.F. & Griffen, E.J. (1989). A selective differential medium for the isolation of *Listeria monocytogenes*. *Letters in Applied Microbiology*, **8**, 95–98.

De Oliveira, M.A., Ribeiro, E.G.A., Bergamini, A. M. M. & De Martinis, E. C. P. (2010). Quantification of *Listeria monocytogenes* in minimally processed leafy vegetables using a combined method based on enrichment and 16S rRNA real-time PCR. *Food Microbiology*, **27**, 19–23.

De Vasconcelos, R.M., de Almeida, A.E.C.C., Hofer, E., da Silva, N.M.M. & Marin, V.A. (2008). Multiplex-PCR serotyping of *Listeria monocytogenes* isolated from human clinical specimens. *Memórias do Instituto Oswaldo Cruz*, **103**, 836–838.

Decatur, A.L. & Portnoy, D.A. (2000). A PEST-like sequence in listeriolysin O essential for *Listeria monocytogenes* pathogenicity. *Science*, **290**, 992–995.

Donnelly, C.W. (1999). Conventional methods to detect and isolate *Listeria monocytogenes*. In: *Listeria, Listeriosis, and Food Safety* (edited by E.T. Ryser & E.H. Marth). Pp. 225–260. New York: Marcel Dekker, Inc

Donnelly, C.W. (2001). *Listeria monocytogenes*: a continuing challenge. *Nutrition Reviews*, **59**, 183–194.

Doumith, M., Buchrieser, C., Glaser, P., Jaquet, C. & Martin, P. (2004a). Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. *Journal of Clinical Microbiology*, **42**, 3819–3822.

Stellenbosch University http://scholar.sun.ac.za

Doumith, M., Cazalet, C., Simoes, N., Frangeul, L., Jacquet, C., Kunst, F., Martin, P., Cossart, P., Glaser, P. & Buchrieser, C. (2004b). New aspects regarding evolution and virulence of *Listeria monocytogenes* revealed by comparative genomics and DNA arrays. *Infection and Immunity*, **72**, 1072–1083.

Ertaş, H.B. & Şeker, E. (2005). Isolation of *Listeria monocytogenes* from fish intestines and RAPD analysis. *Turkish Journal of Veterinary and Animal Sciences*, **29**, 1007–1011.

European Commission (1999). *Opinion of the Scientific Committee on Veterinary Measures Relating to Public Health on Listeria monocytogenes*. European Commission, Health and Consumer Protection Directorate General, Brussels, Belgium.

Erdenlig, S., Ainsworth, A.J. & Austin, F.W. (2000). Pathogenicity and production of virulence factors by *Listeria monocytogenes* isolates from channel catfish. *Journal of Food Protection*, **63**, 613–619.

Ericsson, H., Stålhandske, P., Danielsson-Tham, M.L., Bannerman, E., Bille, J., Jacquet, C., Rocourt, J. & Tham, W. (1995). Division of *Listeria monocytogenes* serovar 4b strains into two groups by PCR and restriction enzyme analysis. *Applied and Environmental Microbiology,* **61**, 3872–3874.

Farber, J. & Addison, C. (1994). RAPD typing for distinguishing species and strains in the genus *Listeria*. *Journal of Applied Bacteriology*, **77**, 242–250.

Farber, J.M., Coates, F. & Daley, E. (1992). Minimum water activity requirements for the growth of *Listeria monocytogenes*. *Letters in Applied Microbiology*, **15**, 103–105.

Farber, J.F. & Peterkin, P.I. (1991). *Listeria monocytogenes*, a food-borne pathogen. *Microbiological Reviews*, **55**, 476–511.

Finney, M. (2000). Pulsed-field gel electrophoresis. In: *Current Protocols in Molecular Biology* (edited by F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith & K. Struhl). Pp. 2.5B.1–2.5B.12. Canada: Wiley and Sons.

Franciosa, G., Tartaro, S., Wedell-Neergaard, C. & Aureli, P. (2001). Characterization of *Listeria monocytogenes* strains involved in invasive and noninvasive listeriosis outbreaks by PCR-based fingerprinting techniques. *Applied and Environmental Microbiology*, **67**, 1793–1799.

Stellenbosch University http://scholar.sun.ac.za

Francis, G.A., Thomas, C. & O'Beirne, D. (1999). The microbiological safety of minimally processed vegetables. *International Journal of Food Science & Technology,* **34**, 1–22.

Galsworthy, S.B., Girdler, S. & Koval, S.F. (1990). Chemotaxis in *Listeria monocytogenes*. *Acta Microbiologica Hungarica*, **37**, 81–85.

Gandhi, M. & Chikindas, M.L. (2007). *Listeria*: a foodborne pathogen that knows how to survive. *International Journal of Food Microbiology*, **113**, 1–15.

Gasanov, U., Hughes, D. & Hansbro, P.M. (2004). Methods for the isolation and identification of *Listeria* spp. and *Listeria monocytogenes*: a review. *FEMS Microbiology Reviews*, **29**, 851–875.

Gedde, M.M., Higgins, D.E., Tilney, L.G. and Portnoy, D.A. (2000). Role of listeriolysin O in cell-to-cell spread of *Listeria monocytogenes*. *Infection and Immunity*, **68**, 999–1003.

George, S.M. & Lund, B.M. (1992). The effect of culture medium and aeration on growth of *Listeria monocytogenes* at pH 4.5. *Letters in Applied Microbiology*, **15**, 49–52.

Gerner-Smidt, P., Hise, K., Kincaid, J., Hunter, S., Rolando, S., Hyytia-Trees, E., Ribot, E.M. & Swaminathan, B. (2006). PulseNet USA: a five-year update. *Foodborne Pathogens and Disease*, **3**, 9–19.

Gombas, D.E., Chen, Y., Clavero, R.S. & Scott, V.N. (2003). Survey of *Listeria monocytogenes* in ready-to-eat foods. *Journal of Food Protection*, **66**, 559–569.

Gouin, E., Mengaud, J. & Cossart, P. (1994). The virulence gene cluster of *Listeria monocytogenes* is also present in *Listeria ivanovii*, an animal pathogen, and *Listeria seeligeri*, a non-pathogenic species. *Infection and Immunity*, **62**, 3550–3553.

Gracieux, P., Roche, S.M., Pardon, P. & Velge, P. (2003). Hypovirulent *Listeria monocytogenes* strains are less frequently recovered than virulent strains on PALCAM and Rapid'*L. mono* media. *International Journal of Food Microbiology*, **83**, 133–145.

Graham, T., Golsteyn-Thomas, E.J., Gannon, V.P. & Thomas, J.E. (1996). Genus- and species-specific detection of *Listeria monocytogenes* using polymerase chain reaction assays targeting the 16S/23S intergenic spacer region of the rRNA operon. *Canadian Journal of Microbiology*, **42**, 1155–1162.

Stellenbosch University http://scholar.sun.ac.za

Graham, T.A., Golsteyn-Thomas, E.J., Thomas, J.E. & Gannon, V.P. (1997). Inter- and intraspecies comparison of the 16S–23S rRNA operon intergenic spacer regions of six *Listeria* spp. *International Journal of Systematic Bacteriology*, **47**, 863–869.

Gray, M., Zadoks, R.N., Fortes, E.D., Dogan, B., Cai, S., Chen, Y., Scott, V.N., Gombas, P.E. & Boor, K.J. (2004). *Listeria monocytogenes* isolates from foods and humans form distinct but overlapping populations. *Applied and Environmental Microbiology,* **70**, 5833–5841.

Greenwood, M., Willis, C., Doswell, P., Allen, G. & Pathak, K. (2005). Evaluation of chromogenic media for the detection of *Listeria* species in food. *Journal of Applied Microbiology*, **99**, 1340–1345.

Groves, R.D. & Welshimer, J. (1977). Seperation of pathogenic from apathogenic *Listeria monocytogenes* by three in vitro reactions. *Journal of Clinical Microbiology*, **5**, 559–563.

Guerra, M.M., Bernardo, F. & McLauchlin, J. (2002). Amplified fragment length polymorphism (AFLP) analysis of *Listeria monocytogenes*. *Systematic and Applied Microbiology*, **25**, 456–461.

Gugnani, H.C. (1999). Some emerging food and water borne pathogens. *Journal of Communicable Diseases*, **31**, 65–72.

Gurtler, V. & Mayall, B.C. (2001). Genomic approaches to typing, taxonomy and evolution of bacterial isolates. *International Journal of Systematic and Evolutionary Microbiology*, **51**, 3–16.

Henry, B.S. (1933). Dissociation of the genus *Brucella*. *Journal of Infectious Diseases,* **52**, 374–402.

Higgins, D.L. & Robinson, B. (1993). Comparison of micro-ID *Listeria* method with conventional biochemical methods for identification of *Listeria* isolated from food and environmental samples: collaborative study. *Journal of the Association of Official Analytical Chemists*, **76**, 831–838.

Hitchins, A.D. (2001). Bacteriological Analytical Manual Online: Chapter 10 Detection and enumeration of *Listeria monocytogenes* in food. [WWW document]. URL http://www.fda.gov/food/scienceresearch/laboratorymethods/bacteriologicalanalyticalmanualbam/ucm071400.htm. 21 October 2010.

Hofer, E. & Hofer, C.B. (2005). Listeriose. In: *Dinâmica das doenças infecciosas e parasitárias* (edited by J Rodrigues Coura). Pp. 1539–1546. Guanabara Koogan, Rio de Janeiro.

Hoffman, A.D. & Weidmann, M. (2001). Comparative evaluation of culture and BAX polymerase chain reaction-based detection methods for *Listeris* spp. and *Listeria monocytogenes* in environmental and raw fish samples. *Journal of Food Protection*, **64**, 1521–1526.

Hood, S.K. & Zottola, E.A. (1997). Adherence to stainless steel by foodborne microorganisms during growth in model food systems. *International Journal of Food Microbiology*, **37**, 145–153.

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

Jacquet, C., Doumith, M., Gordon, J.I., Matin, P.M.V., Cossat, P. & Lecerit, M. (2004). A molecular marker for evaluating the pathogenic potential of foodborne *Listeria monocytogenes*. *Journal of Infectious Diseases,* **189**, 2094–2100.

Jay, J.M. (1996). Foodborne listeriosis. In: *Modern Food Microbiology* (edited by D.R. Heldman). Pp. 478–506. New York: Chapman & Hall.

Jersek, B., Gilot, P., Gubina, M., Klun, N., Mehle, J., Tcherneva, E., Rijpens, N. & Herman, L. (1999). Typing of *Listeria monocytogenes* strains by repetitive element sequence-based PCR. *Journal of Clinical Microbiology*, **37**, 103–109.

Johnson, J.L. & Lattuada, C.P. (1993). Comparison of nucleic acid hybridization assays and biochemical characterization tests for the confirmation of *Listeria monocytogenes*. *Journal of Food Protection*, **56**, 834–840.

Jorgensen, F., Stephens, P.J. & Knochel, S. (1995). The effect of osmotic shock and subsequent adaptation on the thermotolerance and cell morphology of *Listeria monocytogenes*. *Journal of Applied Bacteriology*, **79**, 274–281.

Junttila, J.R., Niemelä, S.I. & Hirn, J. (1988). Minimum growth temperatures of *Listeria monocytogenes* and non-heamolytic *Listeria*. *Journal of Applied Bacteriology*, **65**, 321–327.

Kalmokoff, M.L., Austin, J.W., Wan, X.D., Sanders, G., Banerjee, S. & Farber, J.M. (2001). Adsorption, attachment and biofilm formation among isolates of *Listeria monocytogenes* using model conditions. *Journal of Applied Microbiology,* **91**, 725–734.

Stellenbosch University http://scholar.sun.ac.za

Kalpana, K., Mitra, S. & Muriana, P.M. (2004). Subtyping of *Listeria monocytogenes* by Multilocus Sequence Typing. Research Report. Division of Agricultural Sciences & Natural Resources, Oklahoma State University, Oklahoma, United States of America.

Kathariou, S. (2002). *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *Journal of Food Protection*, **65**, 1811–1829.

Kayal, S., Lilienbaum, A., Poyart, C., Memet, S., Israel, A. and Berche, P. (1999). Listeriolysin O-dependent activation of endothelial cells during infection with *Listeria monocytogenes*: activation of NF-kappa B and upregulation of adhesion molecules and chemokines. *Molecular Microbiology*, **31**, 1709–1722.

Kennedy, J. & Wall, P. (2007). Food safety challenges. In: *Food Safety Handbook: Microbiological Challenges* (edited by M. Storrs, M.-C. Devoluy, P. Cruveiller). Pp. 8–19. France: Bio-Me´ rieux Education.

Keto-Timonen, R.O., Autio, T.J. & Korkeala, H.J. (2003). An improved amplified fragment length polymorphism (AFLP) protocol for discrimination of *Listeria* isolates. *Systematic and Applied Microbiology*, **26**, 236–244.

Kimoto, T., Kawamura, I., Kohda, C., Nomura, T., Tsuchiya, K., Ito, Y., Watanabe, I., Kaku, T., Setianingrum, E. & Mitsuyama, M. (2003). Differences in gamma interferon production induced by listeriolysin O and ivanolysin O result in different levels of protective immunity in mice infected with *Listeria monocytogenes* and *Listeria ivanovii*. *Infection and Immunity*, **71**, 2447–2454.

Kingdon, G.C. and Sword, P. (1970). Effect of *Listeria monocytogenes* hemolysin on phagocytic cells and lysosomes. *Infection and Immunity*, **1**, 356–362.

Klæboe, H., Rosef, O. & Sæbø, M. (2005). Longitudinal studies on *Listeria monocytogenes* and other *Listeria* species in two salmon processing plants. *International Journal of Environmental Health Research*, **15**, 71–77.

Kuhn, M. and Goebel, W. (1998). Host cell signalling during *Listeria monocytogenes* infection. *Microbiology*, **6**, 11–15.

Lachica, R.V. (1996). Hemolytic activity reevaluation of putative non-pathogenic *Listeria monocytogenes* strains. *Applied and Environmental Microbiology,* **62**, 4293–4295.

Lappi, V.R., Thimothe, J., Nightingale, K.K., Gall, K., Scott, V.N. & Wiedmann, M. (2004). Longitudinal studies on *Listeria* in smoked fish plants: Impact of intervention strategies on contamination patterns. *Journal of Food Protection*, **67**, 2500–2514.

Stellenbosch University http://scholar.sun.ac.za

Larsen, H.E. & Seeliger, H.P.R. (1966). A mannitol fermenting *Listeria*: *Listeria grayi* sp. In: *Proceedings of the Third International Symposium on Listeriosis*. Bilthoven, The Netherlands.

Lawrence, L.M. & Gilmour, A. (1995). Characterization of *Listeria monocytogenes* isolated from poultry products and from the poultry-processing environment by random amplification of polymorphic DNA and multilocus enzyme electrophoresis. *Applied and Environmental Microbiology*, **61**, 2139–2144.

Leimeister-Wachter, M., Haffner, C., Domann, E., Goebel, W. & Chakraborty, T. (1990). Identification of a gene that positively regulates expression of listeriolysin, the major virulence factor of *Listeria monocytogenes*. *Proceedings of the National Academy of Science USA*, **87**, 8336–8340.

Liu, D. (2004). Listeria monocytogenes: comparative interpretation of mouse virulence assay. *FEMS Microbiology Letters*, **233**, 159–164.

Liu, D. (2006). Identification, subtyping and virulence determination of *Listeria monocytogenes*, an important foodborne pathogen. *Journal of Medical Microbiology*, **55**, 645–659.

Liu, D., Ainsworth, A.J., Austin, F.W. & Lawrence, M. L. (2003a). Use of PCR primers derived from a putative transcriptional regulator gene for species-specific determination of *Listeria monocytogenes*. *International Journal of Food Microbiology*, **91**, 297–304.

Liu, D., Austin, F.W., Ainsworth, A.J. & Lawrence, M. (2003b). Characterization of virulent and avirulent *Listeria monocytogenes* strains by PCR amplification of putative transcriptional regulator and internalin genes. *Journal of Medical Microbiology*, **52**, 1066–1070.

Liu, D., Lawrence, M.L., Ainsworth, A.J. & Austin, F.W. (2005). Comparative assessment of acid, alkali and salt tolerance in *Listeria monocytogenes* virulent and avirulent strains. *FEMS Microbiology Letters*, **243**, 373–378.

Louie, M., Jayaratne, P., Luchsinger, I., Devenish, J., Yao, J., Schlech, W. & Simor, A. (1996). Comparison of ribotyping, arbitrarily primed PCR, and pulsed-field gel electrophoresis for molecular typing of *Listeria monocytogenes*. *Journal of Clinical Microbiology*, **34**, 15–19.

Lukinmaa, S., Aarnisalo, K., Suihko, M.-L. & Siitonen, A. (2004). Diversity of *Listeria monocytogenes* isolates of human and food origin studied by serotyping, automated ribotyping and pulsed-field gel electrophoresis. *Clinical Microbiology and Infection*, **10**, 562–568.

Stellenbosch University http://scholar.sun.ac.za

Lyytikainen, O., Nakari, U.M., Lukinmaa, S., Kela, E., Nguyen Tran Minh, N. & Siitonen, A. (2006). Surveillance of listeriosis in Finland during 1995 – 2004. *Eurosurveillance* **11**, 82–85.

Mafu, A.A., Roy, D., Goulet, J. & Magny, P. (1990). Attachment of *Listeria monocytogenes* to stainless-steel, glass, polypropylene, and rubber surfaces after short contact times. *Journal of Food Protection*, **53**, 742–746.

Mafu, A.A., Roy, D., Goulet, J. & Savoie, L. (1991). Characterization of physicochemical forces involved in adhesion of *Listeria monocytogenes* to surfaces. *Applied and Environmental Microbiology*, **57**, 1969–1973.

Maiden, M.C., Bygraves, J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., Zhang Q., Zhou, J., Zurth, K., Caugant, D.A., Feavers, I.M., Achtman, M. & Spratt, B.G. (1998). Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Sciences USA*, **95**, 3140–3145.

Manfreda, G., de Cesare, A., Stellan, S., Cozzi, M. & Cantoni, C. (2005). Occurrence and ribotypes of *Listeria monocytogenes* in Gorgonzola cheeses. *International Journal of Food Microbiology*, **102**, 287–293.

McKellar, R.C. (1994). Use of the CAMP test for identification of *Listeria monocytogenes*. *Applied and Environmental Microbiology,* **60**, 4219–4225.

McLauchlin, J., Mitchell, R.T., Smerdon, W.J. & Jewell, K. (2004). *Listeria monocytogenes* and listeriosis: a review of hazard characterization for use in microbiological assessment of foods. *International Journal of Food Microbiology,* **92**, 15–33.

Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M. & Tauxe, R. V. (1999). Food-related illness and death in the United States. *Emerging Infectious Diseases*, **5**, 607–625.

Mead, P. S., Dunne, E.F., Graves, L., Wiedmann, M., Patrick, M., Hunter, S., Salehi, E., Mostashari, F., Craig, A., Mshar, P., Bannerman, T., Sauders, B.D., Hayes, P., Dewitt, W., Sparling, P., Griffin, P., Morse, D., Slutsker, L. & Swaminathan, B. (2006). Nationwide outbreak of listeriosis due to contaminated meat. *Epidemiology and Infection*, **134**, 744–751.

Meloni, D., Galluzzo, P., Mureddu, A., Piras, F., Griffiths, M. & Mazzette, R. (2009). *Listeria monocytogenes* in RTE foods marketed in Italy: Prevalence and automated *Eco*RI ribotyping of the isolates. *International Journal of Food Microbiology*, **129**, 166–173.

Stellenbosch University http://scholar.sun.ac.za

Meng, J. & Doyle, M.P. (1997). Emerging issues in microbiological food safety. *Annual Review of Nutrition*, **17**, 255–275.

Mereghetti, L., Lanotte, P., Savoye-Marczuk, V., Marquet-Van Der Mee, N., Audurier, A. & Quentin, R. (2002). Combined ribotyping and random multiprimer DNA analysis to probe the population structure of *Listeria monocytogenes*. *Applied and Environmental Microbiology,* **68**, 2849–2857.

Montville, T.J. & Matthews, K.R. (2008). *Listeria monocytogenes*. In: *Food Microbiology, An Introduction*, 2nd ed. Pp 173–187. Washington, DC: ASM Press.

Moore, M.A. & Datta, A.R. (1994). DNA fingerprinting of *Listeria monocytogenes* strains by pulsed-field gel electrophoresis. *Food Microbiology,* **11**, 31–38.

Nair, S., Karim, R., Cardosa, M.J., Ismail, G. & Pang, T. (1999). Convenient and versatile DNA extraction using agarose plugs for ribotyping of problematic bacterial species. *Journal of Microbiological Methods*, **38**, 63–67.

Nelson, K., Fouts, D.E., Mongodin, E.F., Ravel, J., DeBoy, R.T., Kolonay, J.F., Rasko, D., Angiuoli, S.V., Gill, S.R., Paulsen, I.T., Peterson, J., White, O., Nelson, W.C., Nierman, W., Beanan, M.J., Brinkac, L.M., Daugherty, S.C., Dodson, R.J., Durkin, A.S., Madupu, R., Haft, D.H., Selengut, J., Van Aken, S., Khouri, H., Fedorova, N., Forberger, H., Tran, B., Kathariou, S., Wonderling, L.D., Uhlinch, G.A., Bayles, D.O., Luchanshy, J.B. & Fraser, C.M. (2004). Whole genome comparisons of serovar 4b and 1/2a strains of the food borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. *Nucleic Acids Research*, **32**, 2386–2395.

Nguyen, Van, J. C., Nguyien, L., Guillemain, R., Amrein, C., Buu-Hoï, A. & Gutmann, L. (1994). Relapse of infection or reinfection by *Listeria monocytogenes* in a patient with heart transplant: usefulness of pulsed-field gel electrophoresis for diagnosis. *Clinical Infectious Diseases*, **19**, 208–209.

Norwood, D.E. & Gilmour, A. (2000). The growth and tolerance to sodium hypochlorite of *Listeria monocytogenes* in a steady-state multispecies biofilm. *Journal of Applied Microbiology,* **88**, 512–520.

Norwood, D.E. & Gilmour, A. (2001). The differential adherence capabilities of two *Listeria monocytogenes* strains in monoculture and multispecies biofilms as a function of temperature. *Letters in Applied Microbiology,* **33**, 320–324.

Stellenbosch University http://scholar.sun.ac.za

O'Donoghue, K., Bowker, K., McLauchlin, J., Reeves, D. S., Bennett, P. M. & MacGowan, A. P. (1995). Typing of *Listeria monocytogenes* by random amplified polymorphic DNA (RAPD) analysis. *International Journal of Food Microbiology*, **27**, 245–252.

Pan, Y., Breidt, Jr, F. & Kathariou, S. (2006). Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. *Applied and Environmental Microbiology*, **72**, 7711–7717.

Parisi, A., Latorre, L., Normanno, G., Miccolupo, A., Fraccalvieri, R., Lorusso, V. & Santagada, G. (2010). Amplified fragment length polymorphism and multi-locus sequence typing for high-resolution genotyping of *Listeria monocytogenes* from foods and the environment. *Food Microbiology*, **27**, 101–108.

Paillard, D., Dubois, V., Duran, R., Nathier, F., Guittet, C., Caumette, P. & Quentin, C. (2003). Rapid identification of *Listeria* species by using restriction fragment length polymorphism of PCR-amplified 23S rRNA gene fragments. *Applied and Environmental Microbiology,* **69**, 6386–6392.

Portnoy, D.A., Chakraborty, T., Goebel, W. and Cossart, P. (1992). Molecular determinants of *Listeria monocytogenes* pathogenesis. *Infection and Immunity*, **60**, 1263–1267.

Proctor, M. E., Brosch, R., Mellen, J.W., Garrett, L.A., Kaspar, C.W. & Luchansky, J.B. (1995). Use of pulsed-field gel electrophoresis to link sporadic cases of invasive listeriosis with recalled chocolate milk. *Applied and Environmental Microbiology*, **61**, 3177–3179.

Ramaswamy, V., Cresence, V.M., Rejitha, J.S., Lekshmi, M.U., Dharsana, K.S., Prasad, S.P. & Vijila, H.M. (2007). *Listeria*: review of epidemiology and pathogenesis. *Journal of Microbiology, Immunology and Infection*, **40**, 4–13.

Ripabelli, G., McLauchin, J. & Threlfall, E. J. (2000). Amplified fragment length polymorphism (AFLP) analysis of *Listeria monocytogenes*. *Systematic and Applied Microbiology*, **23**, 132–136.

Robinson, R. K., Batt, C. A. & Patel, P. D. (editors) (2000). Encyclopedia of Food Microbiology. San Diego, CA: Academic Press.

Roche, S. M., Gracieux, P., Albert, I., Gouali, M., Jacquet, C., Martin, P. M. & Velge, P. (2003). Experimental validation of low virulence in field strains of *Listeria monocytogenes*. *Infection and Immunity*, **71**, 3429–3436.

Rocourt, J. & Grimont, P.A.D. (1983). *Listeria welshimeri* sp. nov. and *Listeria seeligeri* sp. nov. *International Journal of Systematic Bacteriology,* **33**, 866–869.

Stellenbosch University http://scholar.sun.ac.za

Rocourt, J. (1999). The genus *Listeria* and *Listeria monocytogenes*: phylogenetic position, taxonomy, and identification. In: *Listeria, Listeriosis, and Food Safety* (edited by E.T. Ryser & E.H. Marth). Pp. 1–12. New York: Marcel Dekker, Inc.

Rousseaux, S., Olier, M., Lemaître, J.P., Piveteau, P. & Guzzo, J. (2004). Use of PCR-restriction fragment length polymorphism of *inlA* for rapid screening of *Listeria monocytogenes* strains deficient in the ability to invade caco-2 cells. *Applied and Environmental Microbiology,* **70**, 2180–2185.

Ryser, E.T., Arimi, S.M., Bunduki, M.M.-C. & Donnelly, C.W. (1996). Recovery of different *Listeria* ribotypes from naturally contaminated, raw refrigerated meat and poultry products with two primary enrichment media. *Applied and Environmental Microbiology,* **62**, 1781–1787.

Sacchetti, R., Bianucci, F. & Ambrogiani, E. (2003). Detection of *Listeria monocytogenes* in foodstuffs using chromogenic isolation media. *New Microbiology*, **26**, 269–274.

Sado, P.N., Jinneman, K.C., Husby, G.J., Sorg, S.M. & Omiecinski, C.J. (1998). Identification of *Listeria monocytogenes* from unpasteurized apple juice using rapid test kits. *Journal of Food Protection*, **61**, 1199–1202.

Salamina, G., Dalle Donne, E., Niccolini, A., Poda, G., Cesaroni, D., Bucci, M., Fini, R., Maldini, M., Schuchat, A., Swaminathan, B., Bibb, W., Rocourt, J., Birkin, N. & Salmaso, S. (1996). A foodborne outbreak of gastroenteritis involving *Listeria monocytogenes*. *Epidemiology and Infection,* **117**, 429–436.

Salcedo, C., Arreaza, L., Alcalá, B., de la Fuente, L. & Vázquez, J.A. (2003). Development of a multilocus sequence typing method for analysis of *Listeria monocytogenes* clones. *Journal of Clinical Microbiology*, **41**, 757–762.

Sallen, B., Rajoharison, A., Desvarenne, S., Quinn, F. & Mabilat, C. (1996). Comparitive analysis of 16S and 23S rRNA sequences of *Listeria* species. *International Journal of Systematic Bacteriology,* **46**, 669–674.

Sauders, B. D., Schukken, Y., Kornstein, L., Reddy, V., Bannerman, T., Salehi, E., Dumas, N., Anderson, B.J., Massey, J.P. & Wiedmann, M. (2006). Molecular epidemiology and cluster analysis of human listeriosis cases in three U.S. states. *Journal of Food Protection*, **69**, 1680–1689.

Savelkoul, P.H.M., Aarts, H.J.M., de Haas, J., Dijkshoorn, L., Duim, B., Osten, M., Rademaker, J.L.W., Schouls, L. & Lenstra, J.A. (1999). Amplified-fragment length polymorphism analysis: the state of the art. *Journal of Clinical Microbiology,* **37**, 3083–3091.

Stellenbosch University http://scholar.sun.ac.za

Seeliger, H.P.R. (1961). Listeriosis. Basel: Karger.

Seeliger, H.P.R. (1981). Apathogene Listerien: *L. innocua* sp. n. (Seeliger et Schoofs, 1977). *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg.*, I. Abt. I Orig. A., **249**, 487–493.

Seeliger, H.P.R., Rocourt, J., Schrettenbrunner, A., Grimont, P.A.D. & Jones, D. (1984). *Listeria ivanovii* sp. nov. *International Journal of Systematic Bacteriology,* **34**, 336–337.

Seeliger, H. P. R. & Jones, D. (1986). Genus *Listeria* Pirie 1940. In: *Bergey's Manual of Systematic Bacteriology* (edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt). **Vol. 2**. Pp 788–795. Baltimore: Williams & Wilkins.

Shearer, A.E.H., Strapp, C.M. & Joerger, R.D. (2001). Evaluation of a polymerase chain reaction-based system for detection of *Salmonella enteritidis*, *Escherichia coli* O157:H7, *Listeria* spp. and *Listeria monocytogenes* on fresh fruits and vegetables. *Journal of Food Protection,* **64**, 788–795.

Sibelius, U., Schulz, E.C., Rose, F., Hattar, K., Jacobs, T., Weiss, S., Chakraborty, T., Seeger, W. and Grimminger, F. (1999). Role of *Listeria monocytogenes* exotoxins listeriolysin and phosphatidylinositol-specific phospholipase C in activation of human neutrophils. *Infection and Immunity*, **67**, 1125–1130.

Sim, J., Hood, D., Finnie, L., Wilson, M., Graham, C., Brett, M. & Hudson, J.A. (2002). Series of incidents of *Listeria monocytogenes* non-invasive febrile gastroenteritis involving ready-to-eat meats. *Letters in Applied Microbiology,* **35**, 409–413.

Singleton, P. & Sainsbury, D. (1978). *Dictionary of microbiology*. p. 303. New York: John Wiley & Sons.

Sleator, R.D., Gahan, C.G. M. & Hill, C. (2003). A postgenomic appraisal of osmotolerance in *Listeria monocytogenes.* *Applied and Environmental Microbiology*, **69**, 1–9.

Smith, A. & Nelson, R.J. (1999). Capillary electrophoresis of DNA. In: *Current Protocols in Molecular Biology* (edited by F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith & K. Struhl). Pp. 2.8.1–2.8.17. Canada: John Wiley and Sons Inc.

Smith, C.L., Lawrance, S.K., Gillespie, G.A., Cantor, C.R., Weissman, S.M. & Collins, F.S. (1987). Strategies for mapping and cloning macroregions of mammalian genomes. *Methods in Enzymology*, **151**, 461–489.

Sohier, R., Benazet, F. & Piéchaud, M. (1948). Sur un germe du genre *Listeria* apparemment non pathogène. *Annales de l'Institut Pasteur*, **74**, 54–57.

Stellenbosch University http://scholar.sun.ac.za

Spencer, J.A.D. (1987). Perinatal listeriosis. *British Medical Journal*, **295**, 349.

Swaminathan, B., Barrett, T.J., Hunter, S.B., Tauxe, R.V. & Force, C.P.T. (2001). PulseNet: the molecular subtyping network for foodborne bacterial disease surveillance, United States. *Emerging Infectious Diseases,* **7**, 382–389.

Swaminathan, B., Rocourt, J. & Bille, J. (1995). *Listeria*. In: *Manual of Clinical Microbiology* (edited by P.R. Murray, E.J. Baron, M.A. Pfaller, F.C. Ternover & R.H. Yolken). Pp. 341–348. Washington, DC: ASM Press.

Thimothe, J., Nightingale, K.K., Gall, K., Scott, V.N. & Wiedmann, M. (2004). Tracking of *Listeria monocytogenes* in smoked fish processing plants. *Journal of Food Protection*, **67**, 328–341.

Tompkin, R.B. (2002). Control of *Listeria monocytogenes* in the food-processing environment. *Journal of Food Protection*, **65**, 709–725.

Van Zyl, J.L. & Ferreira, S.G. (1995). An overview of the Avocado Industry in South Africa. *South African Avocado Growers' Association Yearbook*, **18**, 23–30.

Vazquez-Boland, J.A., Dominguez, L., Fernandez, J.F., Rodriguez-Ferri, E.F., Briones, V., Blanco, M. & Suarez, G. (1990). Revision of the validity of CAMP tests for *Listeria* identification. Proposal of an alternative method for the determination of haemolytic activity by *Listeria* strains. *Acta Microbiologica et Immunologica Hungarica*, **37**, 201–206.

Vazquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehlan, J. & Kreft, J. (2001). *Listeria* pathogenesis and molecular virulence determinants. *Clinical Microbiology Reviews*, **14**, 584–640.

Vogel, B.F., Fussing, V., Ojeniyi, B., Gram, L. & Ahrens, P. (2004). High-resolution genotyping of *Listeria monocytogenes* by fluorescent amplified fragment length polymorphism analysis compared to pulsed-field gel electrophoresis, random amplified polymorphic DNA analysis, ribotyping, and PCR-restriction fragment length polymorphism analysis. *Journal of Food Protection,* **67**, 1656–1665.

Vines, A. & Swaminathan, B. (1998). Identification and characterization of nucleotide sequence differences in three virulence-associated genes of *Listeria monocytogenes* strains representing clinically important serotypes. *Current Microbiology*, **36**, 309–318.

Walker, S.J., Archer, P. & Banks, J.G. (1990). Growth of *Listeria monocytogenes* at refrigeration temperatures. *Journal of Applied Bacteriology*, **68**, 157–162.

Welshimer, H.J. & Meredith, A.L. (1971). *Listeria murrayi*: a nitrate-reducing mannitol-fermenting *Listeria*. *International Journal of Systematic Bacteriology*, **21**, 3–7.

Welshimer, H.J. (1981). The genus *Listeria* and related organisms. In: *The Prokaryotes: A handbook on Habitats, Isolation, and Identification of Bacteria* (edited by M.P. Starr, H. Stolp, A. Truper, A. Balows & H.G. Schlegel). Pp. 1680–1687. Berlin: Springer.

Wiedmann, M., Bruce, J.L., Keating, C., Johnson, A.E., McDonough, P.L. & Batt, C.A. (1997). Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. *Infection and Immunity*, **65**, 2707–2716.

Wong, A.C.L. (1998). Biofilms in food processing environments. *Journal of Dairy Science*, **81**, 2765–2770.

World Health Organization/Food and Agriculture Organization (WHO/FAO) (2004). Risk assessment of *Listeria monocytogenes* in ready-to-eat foods, MRA Series No. 4 – 5. [WWW document]. URL http://www.who.int/foodsafety/publications/micro/mra_listeria/en/index.html. 23 March 2007.

Yde, M. & Genicot, A. (2004). Use of PFGE to characterize clonal relationships among Belgian clinical isolates of *Listeria monocytogenes*. *Journal of Medical Microbiology,* **53**, 399–402.

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CHAPTER 3

IDENTIFICATION OF *LISTERIA MONOCYTOGENES* FROM AN AVOCADO PROCESSING FACILITY USING CONVENTIONAL AND MOLECULAR TECHNIQUES

Abstract

Listeria monocytogenes is a foodborne pathogen that has been isolated from a variety of food sources. This study aimed to investigate and evaluate the reliability of two different *Listeria*-specific agars, Oxford and RAPID'*L.mono*, as well as species-specific and multiplex PCR to correctly identify 94 isolates obtained from an avocado processing facility representing three production seasons as *Listeria* species and *L. monocytogenes*. All of the isolates were identified as *Listeria* species on both Oxford and RAPID'*L.mono* agar. On the RAPID'*L.mono* agar, 76 of the 94 isolates produced typical colonies of *L. monocytogenes* with the remaining 18 showing typical colonies for *L. innocua* (n=13) and *L. ivanovii* (n=5). The species-specific PCR successfully amplified a 730 base pairs (bp) region of the *hly* gene of 80 of the 94 isolates. For the same 80 isolates the multiplex PCR successfully amplified 800, 517 and 238 bp fragments of the *inlA*, *inlC* and *inlJ* genes, respectively. The remaining 14 isolates included the 13 isolates identified as *L. innocua*, as well as an isolate identified as *L. monocytogenes* on RAPID'*L.mono*. The results obtained on the Oxford agar showed a 100 % positive correlation when compared to the PCR results in identifying *Listeria* species, while the RAPID'*L.mono* had a 4 % false negative result in identifying *L. monocytogenes* compared to the PCR results. The results of this study indicated inconsistencies between the results obtained by conventional and molecular detection methods for the identification of *L. monocytogenes*. Species-specific and multiplex PCR, however, proved useful to accurately detect and identify *L. monocytogenes* in a shorter period of time and could replace the use of conventional agar during identification.

Introduction

Listeria monocytogenes is a rod-shaped, Gram-positive, psychrotrophic, mobile and non-endospore-forming foodborne pathogen (Aureli *et al*., 2000; De Vasconcelos *et al*., 2008; De Oliveira *et al*., 2010). It is a facultative intracellular pathogen and has extreme tolerances to pH, temperature and osmolarity. Due to these extreme tolerances, *L.*

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monocytogenes is able to survive many food-processing regimes and has emerged as an important cause of human foodborne infections. This emergence correlates well with the increase in consumption of ready-to-eat (RTE), heat-and-eat and convenience food products (Liu *et al*., 2007a).

The early *L. monocytogenes* identification methods relied on biochemical and phenotypic characteristics and although it is well documented that unreliable results have been obtained for tests that are based on these characteristics, they are still widely used (Bille *et al*., 1992; Johnson & Lattuada, 1993; Lachica, 1996; Sado *et al*., 1998). These unreliable results can be attributed to the fact that phenotypic characteristics are not only determined by the genotype, but also the environmental conditions (Singleton & Sainsbury, 1978).

Various studies on the detection of *L. monocytogenes* by polymerase chain reaction (PCR) have been published during the past two decades (Wang *et al*., 1992; Volokhov *et al*., 2002; Liu *et al*., 2004). By using primers that target virulence and non-virulence factors the identification of *L. monocytogenes* has become more rapid and sensitive (De Oliveira *et al*., 2010). Although *L. monocytogenes* is pathogenic at the species level, it is composed of an assortment of strains with varied virulence and pathogenicity. For this reason, a method that is both rapid and accurate in determining the identity and the pathogenic potential of *L. monocytogenes* strains would be of extreme importance in the prevention of the spread of listeriosis, recall of food products and allaying consumer concerns (Liu *et al*., 2007a).

The aim of this study was to compare two different *Listeria*-specific agars, Oxford and RAPID'*L.mono*, as well as species-specific and multiplex PCR, both specific for *L. monocytogenes*. Both the agars and PCR techniques were evaluated for their reliability in identifying *Listeria* spp. and *L. monocytogenes* strains isolated from an avocado processing facility.

Materials and methods

Microbial strains, media and growth conditions

A collection of 94 presumptive *L. monocytogenes* strains representing product and environmental samples from an avocado processing facility were evaluated. *Listeria monocytogenes* NCTC 7973 (National Collection of Type Cultures, United Kingdom) was included in the analysis as a "type strain" as *L. monocytogenes* NCTC 10357 is defined as the type strain of *L. monocytogenes* but identifies phenotypically as *L.*

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innocua. *Listeria monocytogenes* NCTC 7973 is recommended as a typical *L. monocytogenes* strain. A *Staphylococcus aureus* subsp. *aureus* isolate was included as the negative control.

Two different growth media, Oxford agar (*Listeria* selective agar base (Oxford formulation) CM0856) (Oxoid, Hampshire, United Kingdom) with the Modified *Listeria* Selective Supplement (Oxford formulation) SR0206 (Oxoid), and RAPID'*L.mono* agar (Bio-Rad Laboratories, Johannesburg, South Africa), were evaluated. Both Oxford and RAPID'*L.mono* agar are selective for *Listeria* species with the latter being chromogenic. Oxford agar is based on the principle of aesculin hydrolysis and RAPID'*L.mono* agar on the detection of phosphatidylinositol phospholipase C (PI-PLC) and the inability of *L. monocytogenes* to ferment xylose. The pure cultures were inoculated in 10 mL brain heart infusion (BHI) broth (Merck, Cape Town, South Africa) and incubated at 37 °C for 48 h. All the subcultured isolates were then streaked on Oxford and RAPID'*L.mono* agar and incubated at 37 °C for 48 h.

DNA isolation

Listeria monocytogenes cultured on BHI agar (Merck) were used for DNA isolation. DNA was isolated using a modified method by Wang & Levin (2005). One colony from each strain was transferred to 250 µL double strength (2X) TZ lysis solution (TZ consists of 2.0% Triton X-100 (Merck) and 2.5 mg mL^{-1} sodium azide (Merck) in 0.1 M Tris-HCl at pH 8.0). The samples were then boiled for 10 min in a waterbath, cooled and centrifuged for 5 min at 10 000 x g. A 200 µL volume of the supernatant was transferred to microcentrifuge tubes and used as DNA template.

Species-specific PCR

For the species-specific PCR assay the primer pair consisting of primer A (5'-CAT TAG TGG AAA GAT GGA ATG-3') and primer B (5'-GTA TCC TCC AGA GTG ATC GA-3') was used to amplify a 730 base pair (bp) region of the *hly* gene (Gouws & Liedemann, 2005). Each PCR reaction was performed in a total reaction volume of 25 µL containing 1 X reaction buffer free from $MgCl_2$ (Super-Therm, supplied by Southern Cross Biotechnologies, Cape Town, South Africa), 1.48 mM $MgCl_2$ (Super-Therm), 1 µL DMSO (Merck), 0.4 mM dNTPs (Southern Cross Biotechnologies), 0.4 µM of each primer, 2.5 U *Taq* DNA polymerase (Super-Therm) and 1 µL of DNA template. The cycling conditions were as follows: hot start at 80 °C for 10 min; initial denaturation at 94 °C for 3 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and

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elongation at 72 °C for 30 s; and a final elongation at 72 °C for 2 min. The PCR was performed in an Eppendorf Mastercycler Personal (Merck, Hamburg, Germany) and the generated amplicons were separated on a 1.5 % (m/v) agarose gel containing ethidium bromide (1.6 µL 100 mL^{-1}). Lambda DNA digested with *EcoR*I and *Hind*III (Fermentas) was used as a size indicator and 0.5 X Tris-borate EDTA (TBE) buffer (5 X TBE buffer consists of 53 g Tris base (Sigma-Aldrich, St. Louis, USA), 27.5 g boric acid (Merck) and 20 ml 0.5 M EDTA (Merck) at pH 8.0) was used. The resulting gel was viewed using an ultraviolet transilluminator (Vilber Lourmat, Marne-La-Vallée, France).

Multiplex PCR

For the multiplex PCR assay three primers pairs were used to amplify a 800 bp, 517 bp and 238 bp fragment of the *inlA*, *inlC* and *inlJ* genes, respectively. The *inlA* primer pair consisted of inlA F (5'-ACG AGT AAC GGG ACA AAT GC-3') and inlA R (5'-CCC GAC AGT GGT GCT AGA TT-3') and was used for species-specific recognition. The *inlC* primer pair consisted of inlC F (5'-AAT TCC CAC AGG ACA CAA CC-3') and inlC R (5'-CGG GAA TGC AAT TTT TCA CTA-3') and the *inlJ* primer pair consisted of inlJ F (5'-TGT AAC CCC GCT TAC ACA GTT-3') and inlJ R (5'-AGC GGC TTG GCA GTC TAA TA-3') and these 2 primer pairs were used for virulence determination (Liu *et al*., 2007b). The PCR was performed in a thermal cycler (Eppendorf) in a total reaction volume of 25 µL containing 1 X reaction buffer free from $MgCl_2$ (Super-Therm), 2.5 mM $MgCl_2$ (Super-Therm), 0.2 mM dNTPs (Southern Cross Biotechnologies), 40 pmol of each *inlA* primer, 30 pmol of each *inlC* primer, 20 pmol of each *inlJ* primer, 0.8 U *Taq* DNA polymerase (Super-Therm) and 1 µL of DNA template. The cycling conditions were: 94 °C for 2 min; 30 cycles of 94 °C for 20 s, 55 °C for 20 s and 72 °C for 50 s; and 72 °C for 2 min. The PCR amplicons were separated on a 1.5 % (m/v) agarose gel containing ethidium bromide (1.6 µL 100 mL^{-1}) in 0.5 X TBE buffer. A 100 bp ladder (Fermentas) was used as a size indicator and the resulting gel was viewed using an ultraviolet transilluminator (Vilber Lourmat).

Results and discussion

Growth media

Oxford and RAPID'*L. mono* agars were evaluated for their ability to correctly identify *Listeria* and *L. monocytogenes* and the results are summarised in Table 1.

Table 1 *Listeria monocytogenes* isolates evaluated and the results obtained for both agars and species-specific and multiplex PCR

Isolate	Origin	Oxford agar	RAPID'*L.mono* agar		Species-specific PCR	Multiplex PCR
			Colony	Media		
A1	Product	+	Blue	Red	+	+
A2	Product	+	Blue	Red	+	+
A3	Product	+	Blue	Red	+	+
A4	Unknown source	+	Blue	Red	+	+
A5	Product	+	Blue	Red	+	+
A6	Product	+	Blue-green	Yellow	+	+
A7	Product	+	Blue-green	Yellow	+	+
A8	Product	+	Blue-green	Yellow	+	+
A9	Product	+	Blue	Red	+	+
A10	Product	+	Blue	Red	+	+
A11	Product	+	Blue	Red	+	+
A12	Product	+	Blue	Red	+	+
A13	Product	+	Blue	Red	+	+
A14	Product	+	Blue	Red	+	+
A15	Ribbon blender	+	Blue	Red	+	+
A16	Ribbon blender	+	Blue-green	Yellow	+	+
A17	Product	+	Blue	Red	+	+
A18	Product	+	Blue	Red	+	+

Table 1 continued

Isolate	Origin	Oxford agar	RAPID'*L.mono* agar		Species-specific PCR	Multiplex PCR
			Colony	Media		
A19	Product	+	Blue	Red	+	+
A20	Product	+	Blue	Red	+	+
A21	Product	+	Blue	Red	+	+
A22	Product	+	Blue	Red	+	+
A23	Product	+	Blue	Red	+	+
A24	Product	+	Blue	Red	+	+
A25	Product	+	Blue	Red	+	+
A26	Product	+	Blue	Red	+	+
A27	Product	+	Blue	Red	+	+
A28	Product	+	Blue	Red	+	+
A29	Product	+	Blue	Red	+	+
A30	Product	+	Blue	Red	+	+
A31	Fruit swab	+	Blue	Red	+	+
A32	Fruit swab	+	Blue	Red	+	+
A33	Fruit swab	+	Blue	Red	+	+
A34	Fruit swab	+	Blue	Red	+	+
A35	Product	+	Blue	Red	+	+
A36	Drain	+	Blue	Red	+	+

Table 1 continued

Isolate	Origin	Oxford agar	RAPID' *L.mono* agar		Species-specific PCR	Multiplex PCR
			Colony	Media		
A37	Product	+	Blue	Red	+	+
A38	Product	+	Blue	Red	+	+
A39	Product	+	Blue	Red	+	+
A40	Product	+	Blue	Red	+	+
A41	Product	+	Blue	Red	+	+
A42	Product	+	Blue	Red	+	+
A43	Product	+	Blue	Red	+	+
B1	Drain	+	Blue	Red	+	+
B2	Product	+	Blue	Red	+	+
B3	Drain	+	Blue	Red	+	+
C1	Product	+	Blue	Red	+	+
C2	Platform buckets	+	Blue	Red	+	+
C3	Product	+	Blue	Red	+	+
C4	Platform bottom	+	Blue	Red	-	-
C5	Floor store room	+	White	Red	-	-
C6	Platform bottom bucket	+	Blue	Red	+	+
C7	Product	+	Blue	Red	+	+
C8	Platform buckets	+	Blue	Red	+	+

Table 1 continued

Isolate	Origin	Oxford agar	RAPID'*L.mono* agar		Species-specific PCR	Multiplex PCR
			Colony	Media		
C9	Platform bottom	+	Blue	Red	+	+
C10	Floor blast freezer	+	Blue	Red	+	+
C11	Floor store room	+	Blue	Red	+	+
C12	Floor blast freezer	+	Blue	Red	+	+
C13	Drain	+	White	Red	-	-
C14	Floor blast freezer	+	White	Red	-	-
C15	Floor blast freezer	+	White	Red	-	-
C16	Floor store room	+	White	Red	-	-
C17	Platform buckets	+	Blue	Red	+	+
C18	Platform bottom	+	Blue	Red	+	+
C19	Platform buckets	+	White	Red	-	-
C20	Table	+	Blue	Red	+	+
C21	Floor blast freezer	+	Blue	Yellow	+	+
C22	Store room cutting	+	White	Red	-	-
C23	Product	+	Blue	Red	+	+
C24	Product	+	Blue	Red	+	+
C25	Old cutting floor	+	White	Red	-	-
C26	Tub filler floor	+	White	Red	-	-

Table 1 continued

Isolate	Origin	Oxford agar	RAPID' *L.mono* agar		Species-specific PCR	Multiplex PCR
			Colony	Media		
C27	Store room floor	+	White	Red	-	-
C28	Drain	+	White	Red	-	-
C29	Blender	+	Blue	Red	+	+
C30	Platform bottom	+	Blue	Red	+	+
C31	Table	+	Blue	Red	+	+
C32	Product	+	Blue	Red	+	+
D1	Drain	+	Blue	Red	+	+
D2	Floor	+	White	Red	-	-
D3	Floor	+	White	Red	-	-
D4	Table	+	Blue	Red	+	+
D5	Table	+	Blue	Red	+	+
D6	Floor	+	Blue	Red	+	+
D7	Boot captive	+	Blue	Red	+	+
D8	Drain	+	Blue	Red	+	+
D9	Drain	+	Blue	Red	+	+
D10	Drain	+	Blue	Red	+	+
D11	Drain	+	Blue	Red	+	+
D12	Tub filler	+	Blue	Red	+	+

Table 1 continued

Isolate	Origin	Oxford agar	RAPID' *L.mono* agar		Species-specific PCR	Multiplex PCR
			Colony	Media		
D13	Floor	+	Blue	Red	+	+
D14	Floor	+	Blue	Red	+	+
D15	Floor	+	Blue	Red	+	+
D16	Hosepipe	+	Blue	Red	+	+
Listeria monocytogenes NCTC 7973	Type strain	+	Blue	Red	+	+
Staphylococcus aureus		+	White	Pink	-	-

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Oxford agar

All of the 94 presumptive *L. monocytogenes* strains, *L. monocytogenes* NCTC 7973 and *Staphylococcus aureus* subsp. *aureus* were positively identified as *Listeria* on Oxford agar, thus a 100 % positive for *Listeria* result. Positive colonies are brown-greenish grey in colour with a black halo surrounding the colony due to the aesculin reaction (Fig. 1) (Greenwood *et al.*, 2005). *Staphylococcus aureus* subsp. *aureus* was thus incorrectly identified as *Listeria* using Oxford agar.

This high positive percentage for Oxford media is as a result of the fact that it was developed to distinguish between different *Listeria* species. Media, including Oxford agar, recommended by reference standard methods detect *Listeria* species by revealing the aesculinase (β-glucosidase) activity. This metabolic enzyme is, however, common to all *Listeria* species and does not distinguish *L. monocytogenes* from the other species (Leclerq, 2004). A second step is therefore needed to differentiate *L. monocytogenes* from other *Listeria* species. The time required (5 – 6 days) and the unreliability of the second step tests, such as biochemical tests and haemolytic activity have led to the development of alternative media such as RAPID*'L.mono*.

RAPID'*L.mono* agar

On the RAPID'*L.mono* agar, 76 of the 94 presumptive *L. monocytogenes* strains analysed, as well as *L. monocytogenes* NCTC 7973 formed blue colonies on a red media background, which is indicative of *L. monocytogenes* (Fig. 2(a)). The blue colonies formed indicate the detection of PI-PLC due to the hydrolysis of X-inositol phosphate and the red media background indicates the non-fermentation of xylose. Five strains (A6, A7, A8, A16, C21) had blue-green colonies on a yellow media background (indicating fermentation of xylose), which is indicative of *L. ivanovii* (Fig. 2(b)) and 13 strains (C5, C13, C14, C15, C16, C19, C22, C25, C26, C27, C28, D2, D3) had white colonies (no hydrolysis of X-inositol phosphate) on a red media background which is indicative of *L. innocua*. *Staphylococcus aureus* subsp. *aureus* had white colonies on a bright pink media background (Fig. 2(c)) which is not identified as a result related to *Listeria* species.

With RAPID*'L.mono* agar, differentiation between the *Listeria* species is possible. The RAPID*'L.mono* medium is based on the detection of PI-PLC, encoded by the *plcA* gene which is part of the *L. monocytogenes* virulence gene cluster. A mutation in this virulence gene cluster could lead to suppression of expression of the *plcA* gene, creating PI-PLC defective mutants and, therefore, result in slow growing colonies with

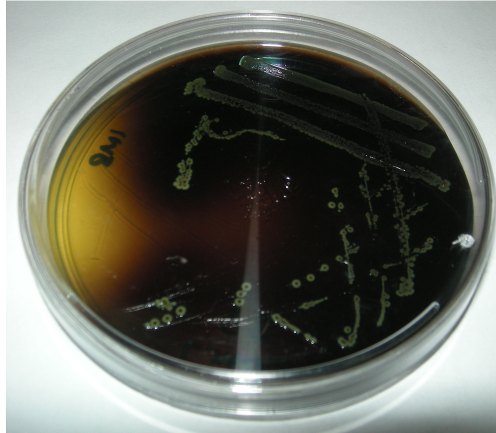
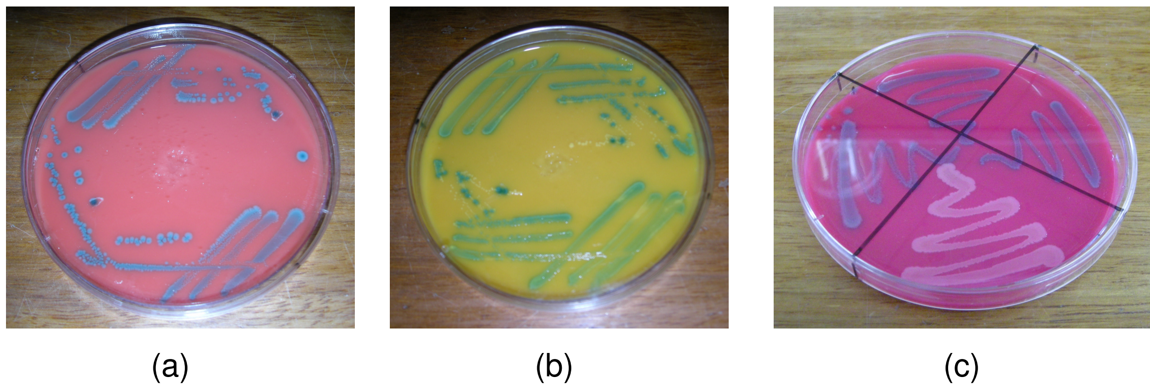


Figure 1 Oxford agar plate with typical *L. monocytogenes* growth.



(a) (b) (c)

Figure 2 RAPID'*L.mono* agar plates with (a) blue colonies on a red media background which is a positive result for *L. monocytogenes* (b) blue-green colonies on a yellow media background, which is a positive result for *L. ivanovii* (c) white colonies on a bright pink media background (bottom of the agar plate) which is an undocumented result.

atypical morphological characteristics (Leclerq, 2004). This observable fact has also been reported by Camilli *et al*. (1991). As these atypical colonies do not meet the typical colony morphology, it would be perceived as negative for *L. monocytogenes* and give rise to false-negative results. Failure to detect these mutant strains should not give rise to public health concerns as these strains are less virulent than normal strains. PI-PLC acts in synergy with listeriolysin O (LLO) to destroy the phagosome in which the bacterium is trapped during infection. If PI-PLC is absent, the bacterium will not be able to escape the phagosome, multiply in the cytosol and infect other cells, resulting in a less virulent strain. However, it should be noted that none of these media can determine the level of virulence of *L. monocytogenes* (Leclerq, 2004).

Oxford versus RAPID'*L.mono* agar

For both the Oxford agar and RAPID*'L.mono* agar all of the 94 strains analysed, as well as *L. monocytogenes* NCTC 7973 (total n=95), were positively identified as members of the genus *Listeria*. Of these 95 strains, 77 (81 %) were positively identified as *L. monocytogenes* on RAPID*'L.mono*. Two chromogenic agars, Agar *Listeria* according to Ottaviani and Agosti (ALOA) and RAPID*'L.mono* were compared to Oxford agar for the detection and enumeration of *Listeria* species in food (Greenwood *et al*., 2005). *Listeria* species were isolated from 63 of the 170 samples investigated, with no significant difference in recovery on the three media. However, it was noted that the recovery of *Listeria* species other than *L. monocytogenes* were significantly poorer on RAPID*'L.mono* than the other two agars. This is a direct result of the selectivity of RAPID*'L.mono* for *L. monocytogenes*. Blue and white colonies which were identified as *L. innocua* and staphylococci, respectively when further confirmatory work was carried out, was also found during the study. Greenwood *et al*. (2005) concluded that visual detection of *L. monocytogenes* and other *Listeria* species were easier on chromogenic media than Oxford agar.

Four commercial agars, including Agar *Listeria* according to Ottaviani and Agosti (ALOA), polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol (PALCAM), Oxford and RAPID*'L.mono* were evaluated for the detection and enumeration of 176 Belgian strains of *L. monocytogenes* from food and human origin (Leclerq, 2004). The results indicated that a combination of these media, one alternative (such as RAPID*'L.mono*) and one aesculin-containing (such as Oxford) should be used to detect and enumerate atypical strains. Gouws & Liedemann (2005) also investigated the effectiveness of both Oxford and RAPID*'L.mono* agars to detect and confirm the presence of *L.*

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monocytogenes. Of the 27 samples tested, 74 % was identified as presumptively positive for *Listeria* on Oxford agar and only 44 % presumptively positive for *L. monocytogenes* on RAPID*'L.mono*. The different percentages obtained for Oxford and RAPID*'L.mono* agar are attributed to the fact that Oxford agar does not differentiate between *Listeria* species (Gouws & Liedemann, 2005). All *Listeria* species demonstrates the same colonial morphology on Oxford agar (Greenwood *et al*., 2005; Gouws & Liedemann, 2005). RAPID*'L.mono* agar is selective for *L. monocytogenes* and therefore colonies presumptively positive for *L. monocytogenes* exhibit a completely different morphology than those for other *Listeria* species. This improves the accuracy of correctly identifying *L. monocytogenes* colonies on RAPID*'L.mono* agar and as a result, a lower positive percentage may be obtained than on Oxford agar.

PCR

Species-specific PCR

The primer pair A and B was selected to amplify a 730 bp region of the *hly* gene of *L. monocytogenes* (Gouws & Liedemann, 2005). The primers successfully recognised and amplified the *hly* gene from 80 of the 94 presumptive *L. monocytogenes* strains evaluated in this study, as well as *L. monocytogenes* NCTC 7973 (Fig. 3), tested in duplicate. Included in these 80 strains were the 5 strains identified as *L. ivanovii* on RAPID*'L.mono* agar. The 14 remaining strains, which included the 13 strains identified as *L. innocua* on RAPID*'L.mono* agar and strain C4, identified as *L. monocytogenes* on RAPID*'L.mono*, as well as *Staphylococcus aureus* subsp. *aureus* resulted in no amplification product. The same primer pair for amplification of the *hly* gene was used to evaluate 27 food samples (Gouws & Liedemann, 2005). Only 37 % of the 27 food samples tested in the study were confirmed to be positive for *L. monocytogenes* by PCR amplification. The PCR results from the study indicated that all of the positive controls for *L. monocytogenes* used in the PCR were identified and eliminated false negatives and false positives obtained by RAPID*'L.mono* and Oxford media, respectively (Gouws & Liedemann, 2005).

In another study, the *hly* gene was also used for PCR amplification of *L.* monocytogenes, but with a different primer pair (Frece *et al*., 2010). The primer pair, *hlyQ* F and *hlyQ* R successfully amplified a 64 bp region of the *hly* gene for 17.3 % of the 180 milk product samples. The results obtained from the study concluded that it was possible to eliminate false positives obtained by conventional methods by PCR and

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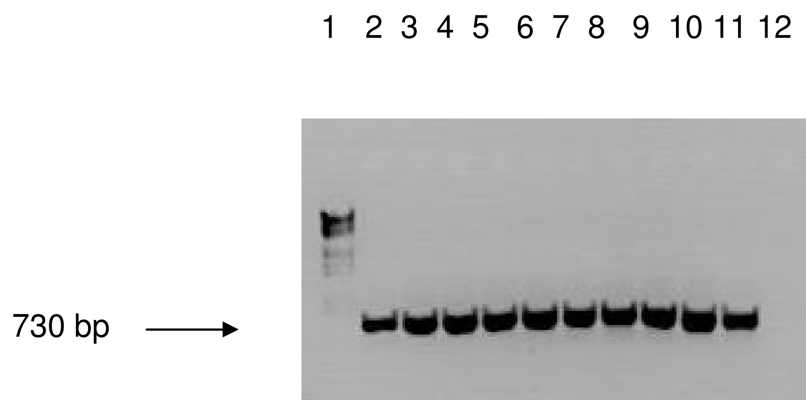

Figure 3 PCR amplification products (730 bp in size) for the species-specific PCR of *L. monocytogenes* strains separated on a 1.5 % (m/v) agarose gel. Lane 1: EcoRI/HindIII digested lambda DNA; Lane 2 - 11: *L. monocytogenes* strain A1, A2, A3, A4, A5, A6, A7, A8, A9, A10; Lane 12: Distilled water (negative control).

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that it is a highly specific, sensitive and rapid technique for the detection of *L. monocytogenes* in food products (Frece *et al*., 2010).

The use of an immunomagnetic separation (IMS) cultivation method for the detection of *L. monocytogenes* in turkey meat samples were investigated during which PCR amplification of the *hlyA* gene was used to confirm the isolates (Bilir Ormanci *et al*., 2008). Two primers, PCRGO and PCRDO were used to amplify a 388 bp region of the *hlyA* gene. Of the 180 samples, *L. monocytogenes* was confirmed in 23 samples by both IMS and PCR. In this case, species-specific PCR was used to verify whether the isolates detected by IMS were *L. monocytogenes* and was, therefore, regarded as a sensitive and specific technique to use.

Multiplex PCR

The *inlA* primer pair, selected for species-specific recognition, successfully amplified a 800 bp fragment of genomic DNA of 80 of the 94 presumptive *L. monocytogenes* strains. These were the same 80 strains which were successfully amplified by the species-specific PCR. *Listeria monocytogenes* NCTC 7973 was also successfully amplified using this primer pair. The *inlC* and *inlJ* primer pairs, selected for virulence determination, also successfully amplified the respective 517 and 238 bp fragments of genomic DNA for the same 80 of the 94 presumptive *L. monocytogenes* strains evaluated in this study, as well as *L. monocytogenes* NCTC 7973 (Fig. 4). This indicates that these 80 strains have the potential to be virulent if these genes are expressed. The remaining 14 strains and *Staphylococcus aureus* subsp. *aureus* gave no amplification with any of the three primer pairs.

Previously, 36 *L. monocytogenes* strains were investigated for species and virulence determination using the same primer pairs (Liu *et al*., 2007b). Pathogenic strains, with the ability to cause mouse mortality, were detected using the *inlA*, *inlC* and/or *inlJ* primers. Naturally non-pathogenic strains were detected by the *inlA* primers but gave negative results with the *inlC* and *inlJ* primers. *Listeria ivanovii* strains were also investigated and 8 out of 10 *L. ivanovii* strains reacted with the *inlC* primers, but not the *inlA* primers and were effectively excluded (Liu *et al*., 2007b). In the present study, the 5 strains that identified as *L. ivanovii* on RAPID*'L.mono* agar, did react with the *inlC* primers, but also the *inlA* and *inlJ* primers, therefore, was identified as *L. monocytogenes* strains with the multiplex PCR.

A multiplex PCR to detect five virulence-associated genes (*prfA*, *plcA*, *hlyA*, *actA* and *iap*) of *L. monocytogenes* were investigated (Kaur *et al*., 2007). Three out of the 10

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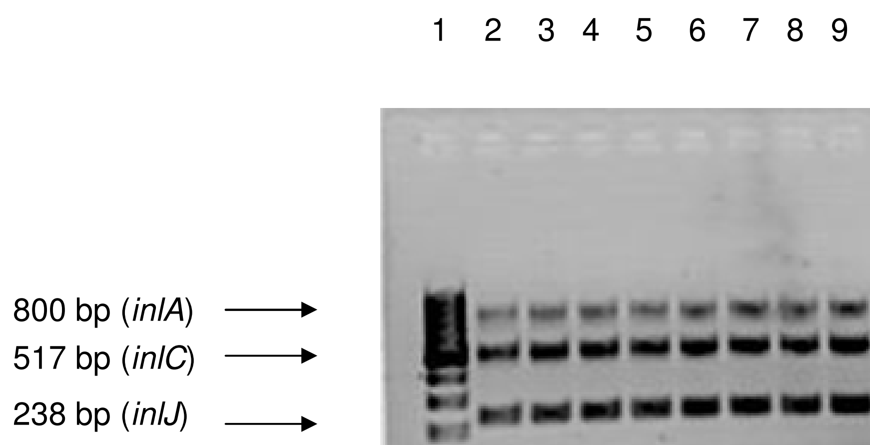

Figure 4 PCR amplification products (800, 517 and 238 bp in size) for the multiplex PCR of *L. monocytogenes* strains separated on a 1.5 % (m/v) agarose gel. Lane 1: 100 bp DNA ladder; Lane 2 – 9: *L. monocytogenes* strain A1, A2, A3, A4, A5, A6, A7, A8.

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Listeria samples analysed were confirmed as *L. monocytogenes* and of these three, two were identified as pathogenic, as they possessed all five genes investigated. *Listeria monocytogenes* strains lacking the *plcA* gene and thus PI-PLC activity were found to be non-pathogenic by *in vivo* tests. They concluded that the *plcA* gene, and the expression thereof, plays an important role as essential virulence determinant in pathogenic *L. monocytogenes* (Kaur *et al.*, 2007).

Of the 94 presumptive *L. monocytogenes* isolates and the "type strain" tested during this study, 100 % were presumptively positive for *Listeria* on Oxford and RAPID*'L.mono* agar, while only 81 % (77 of the 95) were presumptively positive for *L. monocytogenes* on RAPID*'L.mono* agar. All of the 94 presumptive *L. monocytogenes* isolates and the "type strain" were also analysed using species-specific PCR and multiplex PCR. Amplification for a total of 80 of the 94 strains (the same strains), as well as the type strain, was successfully achieved for both PCR assays. When the results of the agars were compared to the PCR results, the Oxford agar had a 100 % positive correlation, as it correctly identified all 80 strains as *Listeria*. On RAPID*'L.mono* agar all 80 strains were also identified as *Listeria* but only 76 strains as *L. monocytogenes*, therefore, a 4 % false negative result in identifying *L. monocytogenes*. Molecular methods are, however, able to detect virulence genes, as shown with the multiplex PCR. As these methods are intrinsically more specific and sensitive than conventional methods, it would be possible to detect atypical strains and give an indication of whether the strains are virulent or not.

Conclusions

The results of this study indicated inconsistencies between the results obtained using different *L. monocytogenes* detection methods. Detection methods that relied on phenotypic characteristics were found to be less sensitive, specific and accurate as those methods that relied on genotypic characteristics.

During this study, RAPID*'L.mono* agar was identified as a relatively sensitive and specific means for detecting and identifying *L. monocytogenes* strains isolated from both food and environmental samples. With Oxford agar, strains were only identified as *Listeria* and further tests were needed to conclusively identify the isolates as *L. monocytogenes*. It is, therefore, recommended that RAPID'L*.mono* be used as a first-step to detect the incidence of *L. monocytogenes* contamination, before further tests are carried out.

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Both the species-specific PCR and multiplex PCR were suitable to accurately detect and identify *L. monocytogenes* strains. Therefore both of these methods would prove useful as a means of confirming the identification of presumptive *L. monocytogenes* colonies obtained on RAPID*'L.mono* agar or even to replace the use of conventional agar during identification, by isolating DNA directly from the product. This replacement will reduce the amount of time, as well as laborious techniques needed to obtain results. This is an important fact to consider as producers of minimally processed food products, with its short shelf-life, have a limited timeframe to obtain microbiological results before releasing their products on the market. The accuracy of the data obtained is also important especially for stringent export regulations and requirements such as the "zero tolerance" level of *L. monocytogenes* in RTE foods in the United States of America where one single *L. monocytogenes* colony will lead to the rejection of the whole batch.

In some cases, the identification of strains as *L. monocytogenes* is not enough, especially in processing plants troubled with a *L. monocytogenes* contamination. In these cases, it will be necessary to determine the source of contamination by distinguishing between the different strains in the raw products, the plant and in the final product (subtyping).

References

Aureli, P., Fiorucci, G.C., Caroli, D., Marchiaro, G., Novara, O., Leone, L. & Salmaso, S. (2000). An outbreak of febrile gastroenteritis associated with corn contaminated by *Listeria monocytogenes*. *The New England Journal of Medicine* **342**, 1236–1241.

Bilir Ormanci, F.S., Erol, I., Ayaz, N.D., Iseri, O. & Sariguzel, D. (2008). Immunomagnetic separation and PCR detection of *Listeria monocytogenes* in turkey meat and antibiotic resistance of the isolates. *British Poultry Science*, **49**, 560–565.

Bille, J., Catimel, B., Bannerman, E., Jacquet, C., Yersin, M.N., Caniaux, I., Monget, D. & Rocourt. J. (1992). API *Listeria*, a new and promising one-day system to identify *Listeria* isolates. *Applied and Environmental Microbiology*, **58**, 1857–1860.

Camilli, A., Goldfine, H. & Portnoy, D.A. (1991). *Listeria monocytogenes* mutants lacking phosphatidylinositol-specific phospholipase C are virulent. *The Journal of Experimental Medicine*, **173**, 751–754.

Stellenbosch University http://scholar.sun.ac.za

Coffey, A., Rombouts, F.M. & Abee, T. (1996). Influence of environmental parameters on phosphatidylcholine phospholipase C production in *Listeria monocytogenes*: a convenient method to differentiate *L. monocytogenes* from other *Listeria* species. *Applied and Environmental Microbiology*, **62**, 1252–1256.

De Oliveira, M.A., Ribeiro, E.G.A., Bergamini, A.M.M. & De Martinis, E.C.P. (2010). Quantification of *Listeria monocytogenes* in minimally processed leafy vegetables using a combined method based on enrichment and 16S rRNA real-time PCR. *Food Microbiology*, **27**, 19–23.

De Vasconcelos, R.M., de Almeida, A.E.C.C., Hofer, E., da Silva, N.M.M. & Marin, V.A. (2008). Multiplex-PCR serotyping of *Listeria monocytogenes* isolated from human clinical specimens. *Memórias do Instituto Oswaldo Cruz*, **103**, 836–838.

Frece, J., Markov, K., Čvek, D., Kolarec, K. & Delaš, F. (2010). Conventional and molecular methods for the routine confirmation of *Listeria monocytogenes* in milk products produced domestically in Croatia. *Journal of Dairy Research*, **77**, 112–116.

Gouws, P.A. & Liedemann, I. (2005). Evaluation of diagnostic PCR for the detection of *Listeria monocytogenes* in food products. *Food Technology and Biotechnology*, **43** (2), 201–205.

Greenwood, M., Willis, C., Doswell, P., Allen, G. & Pathak, K. (2005). Evaluation of chromogenic media for the detection of *Listeria* species in food. *Journal of Applied Microbiology*, **99**, 1340–1345.

Johnson, J.L. & Lattuada, C.P. (1993). Comparison of nucleic acid hybridization assays and biochemical characterization tests for the confirmation of *Listeria monocytogenes*. *Journal of Food Protection*, **56**, 834–840.

Kaur, S., Malik, S.V.S., Vaidya, V.M. & Barbuddhe, S.B. (2007). *Listeria monocytogenes* in spontaneous abortions in humans and its detection by multiplex PCR. *Journal of Applied Microbiology*, **103**, 1889–1896.

Lachica, R.V. (1996). Hemolytic activity reevaluation of putative non-pathogenic *Listeria monocytogenes* strains. *Applied and Environmental Microbiology,* **62**, 4293–4295.

Leclerq, A. (2004). Atypical colonial morphology and low recoveries of *Listeria monocytogenes* strains on Oxford, PALCAM, Rapid'L.mono and ALOA solid media. *Journal of Microbiological Methods*, **57**, 251–258.

Stellenbosch University http://scholar.sun.ac.za

Liu, D., Ainsworth, A.J., Austin, F.W. & Lawrence, M.L. (2004). Use of PCR primers derived from a putative transcriptional regulator gene for species-specific identification of *Listeria monocytogenes*. *International Journal of Food Microbiology*, **91**, 297–304.

Liu, D., Lawrence, M.L., Ainsworth, A.J. & Austin, F.W. (2007a). Toward an improved laboratory definition of *Listeria monocytogenes* virulence. *International Journal of Food Microbiology*, **118**, 101–115.

Liu, D., Lawrence, M.L., Austin, F.W. & Ainsworth, A.J. (2007b). A multiplex PCR for species- and virulence-specific determination of *Listeria monocytogenes*. *Journal of Microbiological Methods*, **71**, 133–140.

Sado, P.N., Jinneman, K.C., Husby, G.J., Sorg, S.M. & Omiecinski, C.J. (1998). Identification of *Listeria monocytogenes* from unpasteurized apple juice using rapid test kits. *Journal of Food Protection*, **61**, 1199–1202.

Singleton, P. & Sainsbury, D. (1978). *Dictionary of microbiology*. New York: John Wiley & Sons. p. 303.

Volokhov, D., Rasooly, A., Chumakov, K. & Chizhikov, V. (2002). Identification of *Listeria* species by microarray-based assay. *Journal of Clinical Microbiology*, **40**, 4720–4728.

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

Wang, S. & Levin, R.E. (2007). Thermal factors influencing detection of *Vibrio vulnificus* using real-time PCR. *Journal of Microbiological Methods*, **69**, 358–363.

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CHAPTER 4

SUBTYPING OF *LISTERIA MONOCYTOGENES* ISOLATES FROM THE AVOCADO PROCESSING ENVIRONMENT USING PCR-RFLP AND PFGE

Abstract

Although *Listeria monocytogenes* is pathogenic at the species level, a variety of subtypes exist containing strains which display varied virulence and pathogenic potential. *Listeria monocytogenes* isolates from an avocado processing facility (n=64) were subtyped using polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) and pulsed-field gel electrophoresis (PFGE). A 733 base pair (bp) fragment of the *inlA* gene was successfully amplified, followed by digestion of the PCR product with the restriction enzymes, *Alu*I and *Tsp*509I. Three different banding patterns were obtained using *Alu*I and two different banding patterns using *Tsp*509I. Subtyping of the isolates using PFGE was carried out by macrorestriction of the genomic DNA with *Apa*I and *Asc*I. The restriction fragments were resolved by PFGE and the fingerprints were classified into four clusters. In the combined analyses, cluster I contained forty-eight isolates (n=48), cluster II 1 isolate (n=1), cluster III fifteen isolates (n=15) and cluster IV 1 isolate (n=1). The PCR-RFLP results had a 98 % correlation with the PFGE results, with the PCR-RFLP being less expensive and results obtainable in a shorter period of time.

Introduction

Listeria monocytogenes is a Gram-positive, facultative, intracellular human pathogen which is predominantly transmitted to humans via the food-borne route (Farber & Peterkin, 1991). It is the cause of the food-borne disease, listeriosis that show symptoms such as meningitis, septicaemia, encephalitis and abortion (Jeffers *et al*., 2001). Different strains of *L. monocytogenes* exist and not all are thought to be pathogenic to humans (Hof & Rocourt, 1992).

Sequence determination of three *L. monocytogenes* virulence genes (*hly*, *iap* and *fla*A) demonstrated that three *L. monocytogenes* lineages exist (Rasmussen *et al*., 1995). The classification of *L. monocytogenes* into these three genetically distinct lineages was confirmed by Wiedmann *et al*. (1997) using the molecular methods of ribotyping and PCR-RFLP. *Listeria monocytogenes* has also been divided into 13 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, 7) upon analysis of group-

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specific *Listeria* O (somatic) and H (flagellar) antigens (Kathariou, 2002). However, serotypes 1/2a, 1/2b, 1/2c and 4b are associated with 95 – 98 % of listeriosis cases (Liu, 2006). Although the conventional serological test has become widely used due to its relative convenience, it lacks specificity and sensitivity and provides results with low discriminatory power and, therefore, does not always provide enough information during epidemiological investigations (Borucki & Call, 2003; Doumith *et al*., 2004).

Currently, molecular methods are being applied for *L. monocytogenes* subtyping which includes amplified fragment length polymorphism (AFLP), pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and ribotyping (Hyytiä-Trees *et al*., 2007). These and other subtyping techniques can be utilised to identify and characterise persistent *L. monocytogenes* contamination in food processing environments due to the adaptation of the bacterium to specific production niches (Vogel *et al*., 2001; Autio *et al*., 2002).

The Centre for Disease Control and Prevention (CDC) in the United States of America, however, regard PFGE as the gold standard technique for the subtyping and successful tracking of *L. monocytogenes* during human listeriosis outbreaks (Graves *et al*., 2005; Gerner-Smidt *et al*., 2006). A national network, PulseNet, coordinated by the CDC allows for the exchange of bacterial PFGE patterns (fingerprints), and has proved to be useful in identifying and controlling human listeriosis outbreaks as a single outbreak may be geographically dispersed (Graves *et al*., 2005; Gottlieb *et al*., 2006; Mead *et al*., 2006).

The aim of this study was to evaluate and subtype the *L. monocytogenes* strains present in an avocado processing facility using molecular techniques (PFGE and PCR-RFLP). Isolates used in this study were collected over a three year period from an avocado processing facility to represent three seasons of production.

Materials and methods

Microbial strains, media and growth conditions

A collection of 64 *L. monocytogenes* isolates obtained from product and environmental samples in an avocado processing facility were analysed. *Listeria monocytogenes* NCTC 7973 (National Collection of Type Cultures, United Kingdom) was included in the analysis as a type strain. Although *L. monocytogenes* NCTC 10357 is defined as the type strain of *L. monocytogenes*, it phenotypically identifies as *L. innocua* and, therefore, *Listeria monocytogenes* NCTC 7973 is recommended as a typical *L.*

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monocytogenes strain (Jones & Seeliger, 1983). *Listeria monocytogenes* isolates were retrieved from glycerol stocks kept at -80 °C. A volume of 100 µl was inoculated in 10 ml brain heart infusion (BHI) broth (Merck, Cape Town, South Africa) and incubated at 37 °C for 48 h. The isolates were then streaked on BHI agar and incubated at 37 °C for 48 h.

DNA isolation

A modified method by Wang & Levin (2005) was used to isolate DNA. One colony from each strain was transferred to 250 µl double strength (2X) TZ lysis solution (TZ consists of 2.0% Triton X-100 (Merck) and 2.5 mg ml^{-1} sodium azide (Merck) in 0.1 M Tris-HCl pH 8.0) in boil-proof microcentrifuge tubes. The samples were then boiled for 10 min, cooled down and centrifuged for 5 min at 10 000 x g. A volume of 200 µl of the supernatant was transferred to sterile microcentrifuge tubes and used as DNA template in the PCR amplification reactions.

PCR-RFLP

For the PCR assay the primer pair consisting of seq01 (5'-AAT CTA GCA CCA CTG TCG GG-3') and seq02 (5'-TGT GAC CTT CTT TTA CGG GC-3') was used to amplify a 733 base pair (bp) fragment of the *inlA* gene (Rousseaux *et al.*, 2004). Each PCR reaction was performed in a total reaction volume of 50 µL containing 1 X reaction buffer free from $MgCl_2$ (Super-Therm, supplied by Southern Cross Biotechnologies, Cape Town, South Africa), 1.5 mM $MgCl_2$ (Super-Therm), 0.8 mM dNTPs (Southern Cross Biotechnologies), 0.5 µM of each primer, 1.25 U *Taq* DNA polymerase (Super-Therm) and 2 µL of DNA template. The PCR was performed in an Eppendorf Mastercycler Personal (Merck, Hamburg, Germany) and the cycling conditions were as follows: initial denaturation at 94 °C for 4 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 1 min and elongation at 72 °C for 2.5 min; and a final elongation at 72 °C for 7 min.

Each restriction digestion was performed in a total reaction volume of 30 µL containing either 1 U FastDigest *Alu*I or FastDigest *Tsp*509I (Fermentas, supplied by Inqaba Biotechnical Industries, Pretoria, South Africa), 10 µL amplicon, 2 µL FastDigest Green buffer and 17 µL distilled water. The *Alu*I samples were incubated at 37 °C for 10 min and the *Tsp*509I samples at 65 °C for 10 min. Digestion products were then separated on a 5 % agarose gel containing ethidium bromide (1.6 µL 100 mL^{-1}) for 1 h at 100 V. A 100 bp ladder (Fermentas) was used as a size indicator and 1 X Tris-borate

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EDTA (TBE) buffer was used as the running buffer. The resulting gel was viewed using an ultraviolet transilluminator (Vilber Lourmat, Marne-La-Vallée, France).

PFGE

Preparation of agarose plugs

Sterile swabs moistened with sterile TE buffer (10 mM Tris:1 mM EDTA, pH 8.0) were used to transfer colonies from the BHI agar plates to sterile McCartney bottles containing 4 mL TE buffer. The optical density (OD) at 610 nm was measured using the Beckman Coulter DU 530 Life science UV/Vis spectrophotometer (Beckman Instruments Inc., Fullerton, California, USA) and adjusted until a reading of 1.00 was obtained for each sample by adding TE buffer or cells. This was done to ensure approximately equal concentrations of cells in the plugs. A 200 µL volume of the cell suspensions (OD = 1.00) were transferred to microcentrifuge tubes and 10 µL lysozyme (20 mg mL^{-1}) (Merck) was added to each tube, the tube was gently mixed and incubated at 55° – 60 °C for 10 – 20 min in a water bath. A 10 µL volume of a 20 mg mL^{-1} Proteinase K solution (Merck) was added to each tube and gently mixed with the pipet tip. A 200 µL volume of melted 1 % SeaKem Gold agarose (Whitehead Scientific, Cape Town, South Africa) was added to the 220 µL cell suspension, mixed and immediately dispensed into plug moulds (Bio-Rad Laboratories, Johannesburg, South Africa). The plugs were allowed to set at room temperature for 15 min. The plugs were pressed out into 5 mL Proteinase K/Cell Lysis Buffer (5 mL Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1 % Sarcosyl) and 25 µL Proteinase K stock solution (20 mg mL^{-1})) and incubated in a 54 °C shaker water bath for 2 h with constant agitation. The Proteinase K/Cell Lysis Buffer was replaced with 15 mL sterile TE buffer and incubated in a 50 °C shaker water bath for 30 min. This washing procedure was repeated three times. Plugs were then transferred to 5 mL sterile TE buffer and stored at 4 °C.

Restriction digestion

A sterile scalpel was used to cut a 2.0 – 3.0 mm piece of the *L. monocytogenes* embedded agarose plug. This plug was placed in a microcentrifuge tube containing 200 µL 1 X Buffer B or 1 X Buffer Tango (Fermentas Life Sciences) for digestion with *Apa*I or *Asc*I (*Sgs*I) (Fermentas Life Sciences), respectively and left to equilibrate at room temperature for 15 min. The buffer was removed and 200 µL restriction enzyme solution (20 µL 10 X Buffer B, 2 µL BSA, 50 U *Apa*I and 173 µL ddH_2O for *Apa*I digestion or 20 µL 10 X Buffer Tango, 2 µL BSA, 40 U *Asc*I and 174 µL ddH_2O for *Asc*I digestion) was

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added to cover the plugs. Plugs were incubated at 37 °C for 3 h in a water bath. After enzyme digestion, the restriction enzyme solution was removed and the plugs were immediately loaded for gel electrophoresis.

Pulsed-field gel electrophoresis

The restricted plugs were loaded into the wells of a 1 % Megabase agarose gel (Bio-Rad Laboratories) with 0.5 X Tris-borate EDTA (TBE Buffer) and the wells filled with melted 1% SeaKem Gold agarose (Whitehead Scientific) and left to set at room temperature for 15 min. A CHEF DRII system (Bio-Rad Laboratories) was used for the electrophoretic separation. Three litres of 0.5 X TBE buffer was poured into the chamber and allowed to cool to 14 °C. The current-switching parameters were as follows: 4 s initial switch time, 40 s final switch time, 200 V (6 V cm^{-2}), buffer temperature at 14 °C and a total run time of 18 h. After electrophoresis the gel was removed and stained in 500 mL dH_2O containing 40 µL ethidium bromide (10 mg mL^{-1}) for 30 min, followed by destaining in 500 mL dH_2O for 2 h. The separated PFGE fragments were visualised under an ultraviolet transilluminator (Vilber Lourmat) and the captured images were used for analysis.

Analysis of PFGE-CHEF gel images

Captured gel images were analysed using BioNumerics version 2.5 (Applied Maths, Sint-Martens-Latem, Belgium). Groupings of the PFGE fingerprints were performed by means of the Dice similarity coefficient (s_D) and the unweighted pair-group method using arithmetic averages clustering algorithm (UPGMA) (Sneath & Sokal, 1973). A *L. monocytogenes* isolate used as reference standard was included in every gel.

Results and discussion

PCR-RFLP

The primer pair, seq01 and seq02, was selected to amplify a 733 bp fragment of the *inlA* gene of *L. monocytogenes* (Rousseaux *et al.*, 2004). The primers successfully amplified the *inlA* gene of all 64 *L. monocytogenes* isolates evaluated in this study, as well as the type strain NCTC 7973. Restriction digestion of these PCR amplicons with *Alu*I yielded three different profiles or subtypes, containing 7 to 8 bands that range in size from 44 to 241 bp for all the *L. monocytogenes* isolates (Fig. 1). Most of the 64 isolates were associated with *Alu*I PCR-RFLP profiles I and II. Fifty isolates of product

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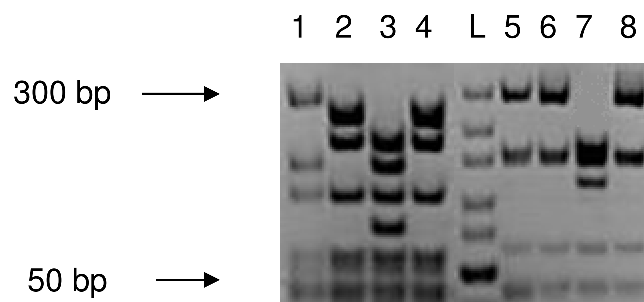

Figure 1 Restriction digestion products for the PCR-RFLP of *L. monocytogenes* isolates separated on a 5 % (m/v) agarose gel. Lane 1 – 4: *Alu*I digests of isolates 43 (*Alu*I profile III), 61 (*Alu*I profile I), 62 (*Alu*I profile II), 64 (*Alu*I profile I); Lane L: 50 bp DNA ladder; Lane 5 – 8: *Tsp*509I digests of isolates 43 (*Tsp*509I profile I), 61 (*Tsp*509I profile I), 62 (*Tsp*509I profile II), 64 (*Tsp*509I profile I).

and environmental origin, as well as the type strain were grouped in *Alu*I profile I and 13 isolates (also of both product and environmental origin) were grouped in *Alu*I profile II. Only one environmental isolate obtained from the drain in the avocado processing plant, was associated with *Alu*I PCR-RFLP profile III (Table 1).

Restriction digestion with *Tsp*509I yielded two profiles or subtypes with four bands each ranging in size from 43 to 281 bp for the 64 *L. monocytogenes* isolates and type strain (Fig. 1). Fifty one isolates, as well as the type strain, were grouped in *Tsp*509I profile I and 13 isolates were grouped in *Tsp*509I profile II. In this case, the single isolate that was associated with PCR-RFLP profile III of the *Alu*I digestion was grouped with those of profile I of the *Tsp*509I digestion (Table 1). The rest of the isolates were grouped in identical groups using both enzymes. Therefore, three composite profiles were generated from the different combinations of PCR-RFLP profiles obtained with both restriction enzymes. Fifty isolates, as well as the type strain were grouped in composite profile A (characterised by *Alu*I profile I and *Tsp*509I profile I). Thirteen isolates were grouped in composite profile D (characterised by *Alu*I profile II and *Tsp*509I profile II) and one isolate in composite profile E (characterised by *Alu*I profile III and *Tsp*509I profile I) (Table 1). From these results, it can be concluded that both restriction enzymes *Alu*I and *Tsp*509I produced profiles that are discriminatory, but with *Alu*I being the most discriminatory enzyme.

The primer pair and restriction enzymes for PCR-RFLP analysis of *L. monocytogenes* used in this study were the same as those used by Rousseaux *et al.* (2004) and Tamburro *et al.* (2010) to compare the PCR-RFLP profiles of *L. monocytogenes* isolates from one factory and *L. monocytogenes* isolates from various factories and humans. Rousseaux *et al.* (2004) performed PCR-RFLP analysis on 37 *L. monocytogenes* strains isolated from various origins, including human and food sources. They obtained five different *Alu*I PCR-RFLP profiles with 8 to 11 bands ranging in size from 15 to 241 bp and three different *Tsp*509I profiles with 7 to 11 bands ranging in size from 8 to 281 bp. The different combinations of PCR-RFLP profiles obtained by both enzymes generated five composite profiles. Most of the isolates were grouped in composite profiles A and B, with profile A corresponding to the composite profile A obtained in this study and profile B not obtained in this study. Interestingly, Rousseaux *et al.* (2004) also found only one isolate, also of environmental origin, grouped in composite profile E (corresponding to composite profile E in this study). As was the case for this study, they found no correlation between the composite profiles and the origin of the isolates.

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Table 1 PCR-RFLP composite profiles

Composite Profile	*Alu*I Profile	*Tsp*509I Profile
A (n=51)	I (n=51)	I (n=52)
D (n=13)	II (n=13)	II (n=13)
E (n=1)	III (n=1)	I (n=52)

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A study by Tamburro *et al.* (2010) reported similar results when analysing 105 *L. monocytogenes* isolates from humans, food and environmental origin in Italy. They also found five different profiles for the *Alu*I digestion and three different profiles for the *Tsp*509I digestion. For the composite profiles, five different profiles were identified, as reported by Rousseaux *et al.* (2004), but a sixth, undefined composite profile was also detected for two isolates of food origin. Most of the isolates (n=72) were grouped in composite profile D (corresponding to composite profile D of Rousseaux *et al.* (2004) and in this study) of which 68 isolates were of food origin.

Although different results were obtained by Rousseaux *et al.* (2004), Tamburro *et al.* (2010) and this study, it is interesting to note that the majority of strains evaluated in this study grouped in composite profiles A and D, corresponded to the predominant composite profiles obtained for the strains of both Rousseaux *et al.* (2004) (composite profile A) and Tamburro *et al.* (2010) (composite profile D).

PFGE

Clustering analysis was done on the PFGE fingerprints obtained using the restriction enzymes, *Apa*I and *Asc*I, independently and combined. All three analyses (*Apa*I, *Asc*I, and *Apa*I and *Asc*I combined) showed that the isolates were grouped into four clusters. The clustering analysis of the fingerprints obtained with restriction enzyme *Apa*I (cluster I contains 48 isolates, cluster II one isolate, cluster III 15 isolates and cluster IV one isolate) and the respective s_D-values are indicated in Fig. 2. The s_D-value is the similarity value and an indication of the relative correlation between the strains of one cluster with those of another cluster. The strains of cluster I were grouped together with a s_D = 82.8 % and grouped with the strain from cluster II (isolate 43) with a s_D = 56.2 %. This s_D-value was expected to be lower as the fingerprint of isolate 43 differed from the strains of cluster I and thus showed a lower correlation. The strains of cluster III were grouped together with a s_D = 70.2 % and grouped together with those of cluster I and II at s_D = 44.3 %. Cluster IV, which contained only the one strain (isolate 65), were grouped with clusters I, II and III at s_D = 28.9 %. This very low s_D-value was also expected as the fingerprint of isolate 65 differed the most from those of clusters I, II and III.

The clustering analysis of the fingerprints obtained with restriction enzyme *Asc*I is indicated in Fig. 3. The fingerprints obtained with *Asc*I were not as differentiating as those obtained by *Apa*I digestion and showed more differences in banding pattern. Although the fingerprints that were clustered together had identical prominent, high

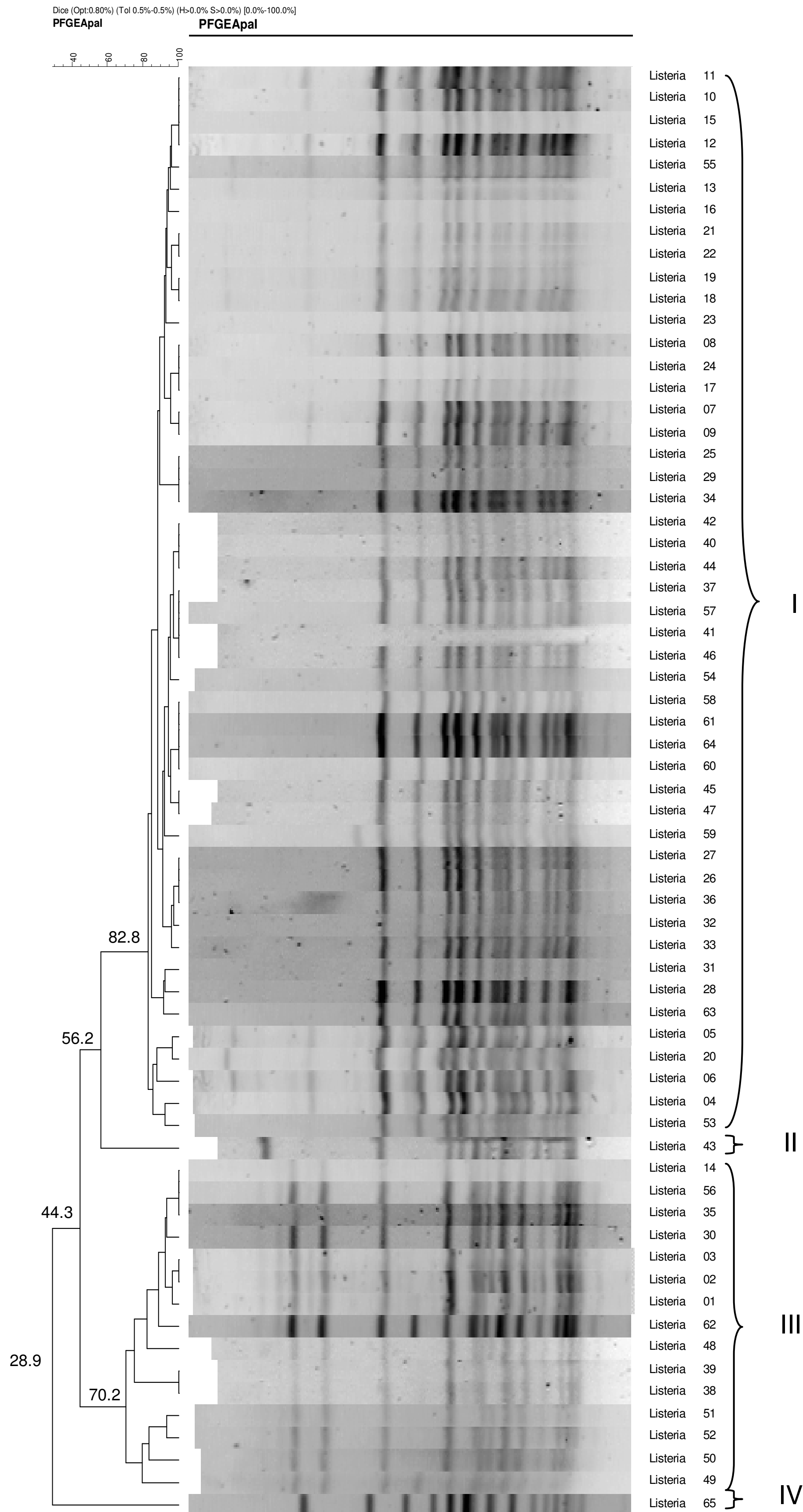

Figure 2 Dendrogram obtained by UPGMA analysis of the similarity value (s_D) of the PFGE fingerprints with the restriction enzyme *Apa*I of 65 *L. monocytogenes* isolates.

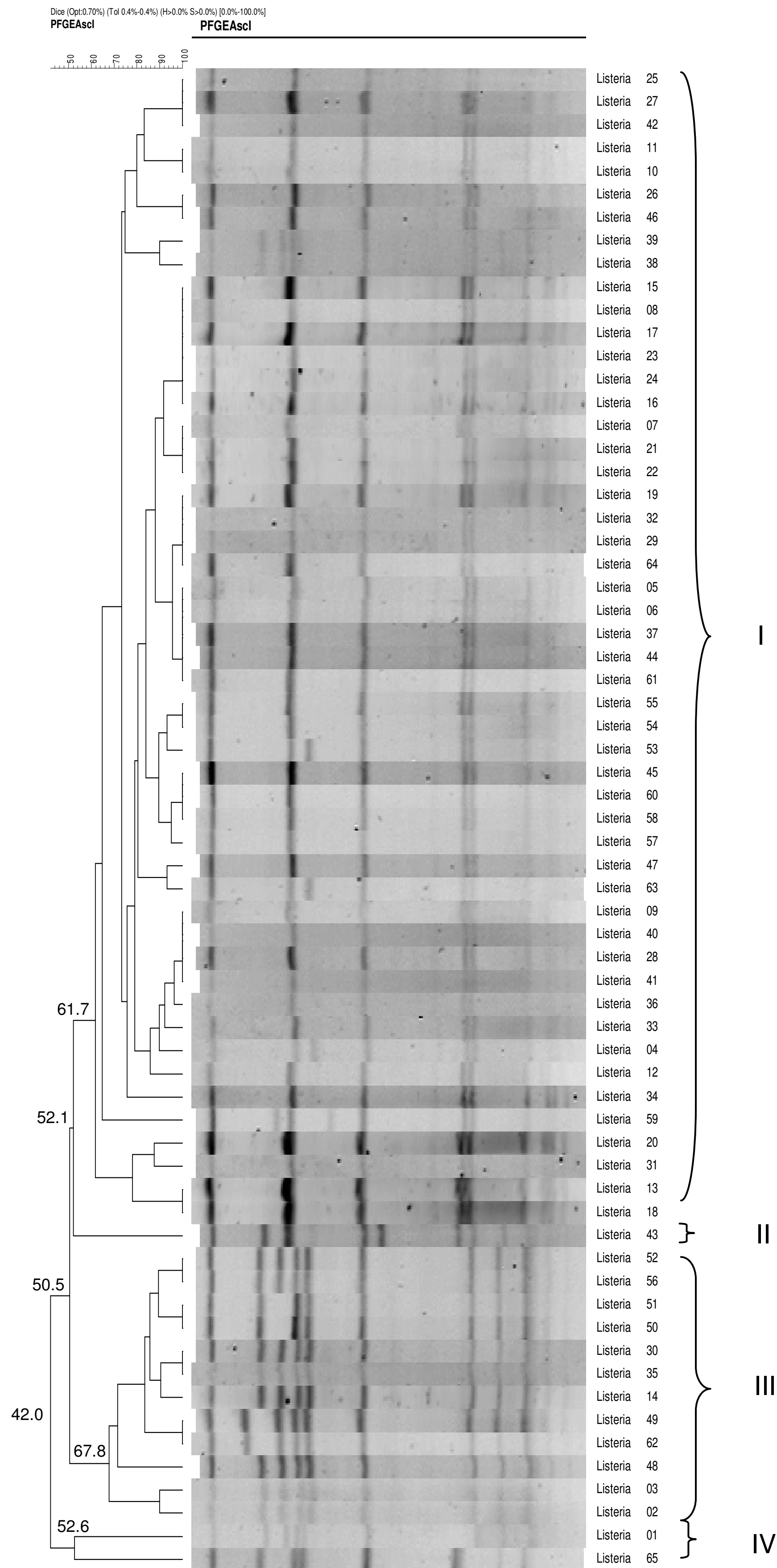

Figure 3 Dendrogram obtained by UPGMA analysis of the similarity value (s_D) of the PFGE fingerprints with the restriction enzyme *Asc*I of 65 *L. monocytogenes* isolates.

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concentration bands, a difference of up to three less prominent bands was observed. Cluster I contained 50 isolates, cluster II contained one isolate, cluster III contained 12 isolates and cluster IV two isolates. The strains of cluster I were grouped together with a s_D = 61.7 % and with the strain from cluster II (isolate 43) with a s_D = 52.1 %. The strains of cluster III were grouped together with a s_D = 67.75 % and grouped together with those of cluster I and II strains at s_D = 50.5 %. Cluster IV strains grouped together with a s_D = 52.63 % and grouped with clusters I, II and III at s_D = 42.01 %. All of these s_D-values are in the same range and serves as an indicator of the less discriminating results obtained using the enzyme *Asc*I for DNA digestion.

The combined fingerprint clustering analysis of the *Apa*I and *Asc*I PFGE profiles (Fig. 4) also revealed four clusters. Cluster I containing 48 isolates grouped together with a s_D = 67.3 %. This percentage was lower than the value obtained for the isolates of *Apa*I cluster I due to the differences in bands of the *Asc*I fingerprints. Cluster II, which consisted of only one isolate (isolate 43) grouped together with the strains of cluster I with a s_D = 46.5 %. Cluster III containing 15 isolates grouped together with a s_D = 59.1 % and with the isolates of cluster I and II with a s_D = 42.9 %. Cluster IV consisting only of isolate 65 (type strain) grouped with cluster I, II and III isolates at s_D = 32.1 %. It can therefore be concluded that three subtypes of *L. monocytogenes* were present in the processing facility as they were resolved into three different clusters with the PFGE analysis.

The *Asc*I clustering analysis clustered isolate 1 together with isolate 65 in cluster IV (Fig. 3) whereas in the *Apa*I and combined clustering analysis (Figs. 2 and 4) isolate 1 was clustered together with the isolates of cluster III, with isolate 65 being the only isolate in cluster IV. However, due to the differences in fingerprints obtained with *Asc*I, this clustering of isolate 1 and 65 in the same group does not necessarily indicate a clonal relationship between the two isolates as they were resolved in different clusters in the combined analyses.

All of the isolates grouped in cluster I with *Apa*I digestion, were also grouped in cluster I of the *Asc*I clustering analysis. However, two isolates, isolates 38 and 39, were grouped in cluster III in the *Apa*I dendrogram (Fig. 2) but grouped in cluster I in the *Asc*I dendrogram (Fig. 3). In the combined dendrogram (Fig. 4), these isolates were resolved in cluster III. This could be the result of the different fingerprints obtained, however, according to the Tenover criteria (Tenover, 1995) isolates are still considered to be closely related if they exhibit one to three bands difference in the PFGE fingerprints.

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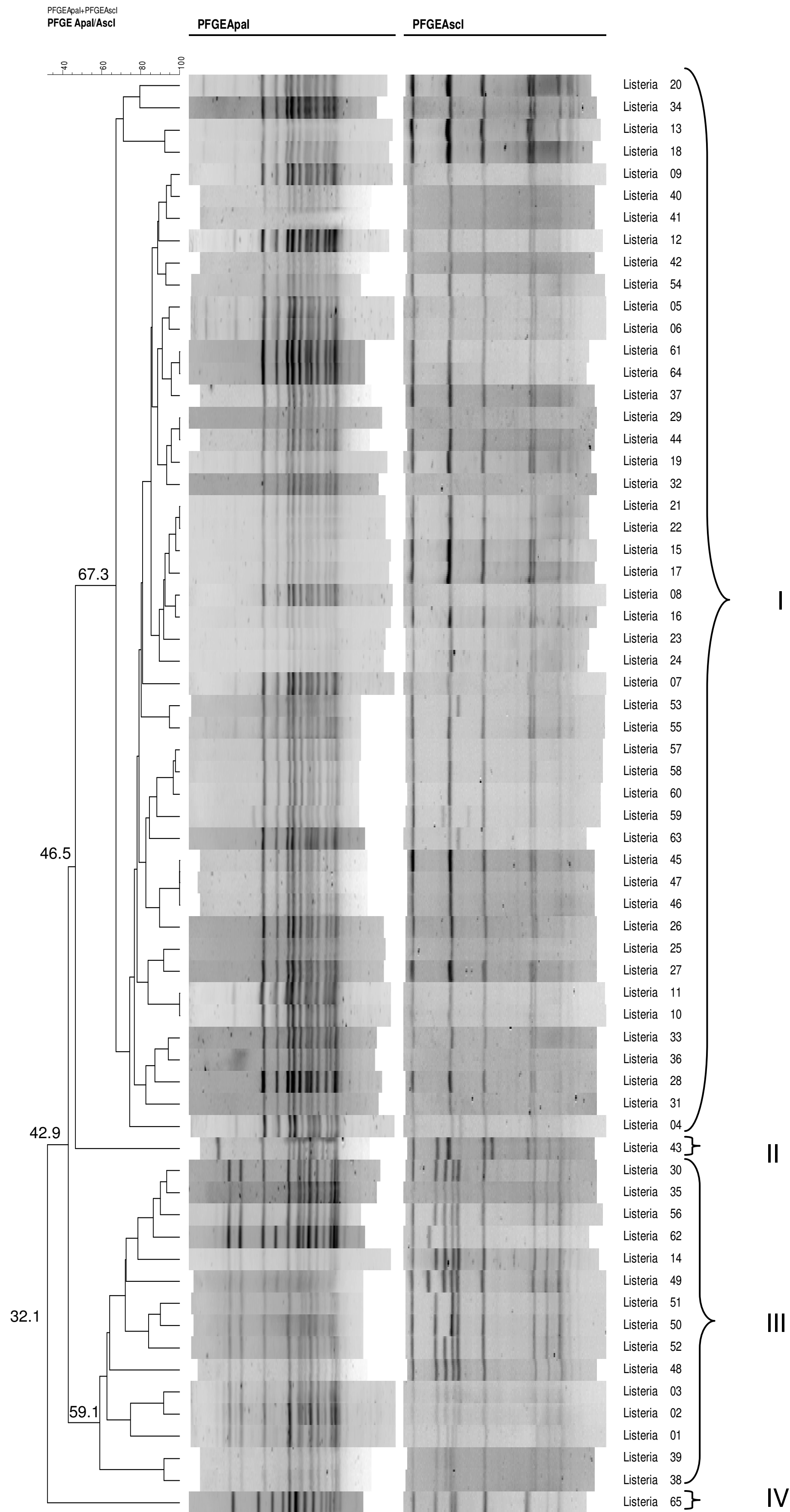

Figure 4 Dendrogram obtained by UPGMA analysis of the similarity value (s_D) of the combined PFGE fingerprints with the restriction enzymes *Apa*I and *Asc*I of 65 *L. monocytogenes* isolates.

Both clusters I and III of all three clustering analyses consisted of product and environmental isolates and, therefore, no correlation could be identified between the clusters and the origin of the isolates. Both of these clusters also contained isolates from all three seasons which might indicate a persistency of strains in the processing facility. Cluster II contained isolate 43, an environmental isolate isolated from the drain and cluster IV contained isolate 65, which is the *L. monocytogenes* type strain NCTC 7973. It is interesting that the type strain was grouped in its own cluster. However, this strain was not isolated from the processing facility and would, therefore, not be clonally related to the strains present in the processing facility. It should, however, have similar or common bands related to the fingerprints as it is also a *L. monocytogenes* strain. In the fingerprints of both *Apa*I and *Asc*I, isolate 65 does share more than one common band with the other strains and thus indicates a relative relationship with the other isolates.

In Italy, a study was conducted to investigate type diversity and distribution of *L. monocytogenes* isolates collected from foods over a five year period. Three hundred *L. monocytogenes* isolates were subtyped by serotyping and PFGE. Conventional serotyping was unable to type 5 % of the isolates, while all of the isolates were subtyped by PFGE generating unique and shared profiles. Some of the profiles obtained were retrieved from isolates from different years indicating a possible persistency of *L. monocytogenes* in certain foods and specific processing facilities (Nucera *et al.*, 2010). Nucera *et al.* (2010) concluded that the designing of a broad typing database in Europe (such as PulseNet in the United States) could be used for epidemiological investigations, as well as assisting with the understanding of *L. monocytogenes* strain diversity.

Fugett *et al.* (2007) investigated PFGE type diversity on a total of 495 *L. monocytogenes* strains from foods, human clinical cases, ruminant farms and urban and natural environments. In this study, PFGE using two enzymes (*Apa*I and *Asc*I) exhibited a higher discriminatory power than *Eco*RI ribotyping. Some of the PFGE profiles obtained showed significant associations with specific sources (human clinical cases and foods), as well as recurrent PFGE profiles each specific to a single processing facility, indicating persistent isolates in the facilities. They concluded that PFGE is highly recommended for the subtyping of *L. monocytogenes* and that some of the *L. monocytogenes* PFGE profiles could be linked to specific sources (Fugett *et al.*, 2007).

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Similarities were observed when the *Alu*I PCR-RFLP results obtained in this study (Fig. 1 and Table 1) were compared to the combined *Apa*I and *Asc*I clustering analysis results obtained by PFGE (Fig. 4). All of the isolates were grouped in identical groups except for isolate 65, the *L. monocytogenes* type strain NCTC 7973. In the *Alu*I PCR-RFLP results the type strain was grouped together with the isolates of the *Alu*I profile I, which corresponds with the cluster I grouping obtained with PFGE. However, in the PFGE results the type strain was grouped in its own group, cluster IV. This might be an indication that although there is a 98 % correlation between the PCR-RFLP and PFGE results, some differences between *L. monocytogenes* strains are not located at the *inlA* gene, which was the target gene for the PCR-RFLP, but elsewhere in the genome. As PFGE is a method that use the whole genome of the micro-organism investigated, it has a higher probability of identifying differences between the strains.

Conclusions

The results of this study indicated that both molecular subtyping methods, PCR-RFLP and PFGE, were sensitive and specific enough to investigate the diversity among the *L. monocytogenes* isolates. The PCR-RFLP results showed a 98 % correlation with the PFGE results, with the PCR-RFLP being less expensive and results obtainable in a shorter period of time. Although more than three days are needed to acquire PFGE results, the results obtained indicate differences in the whole genome whereas PCR-RFLP indicate differences in a specific region of a single gene. As this study indicates there was a 98 % correlation between these two methods and the specific region of the gene chosen for the PCR-RFLP method is highly differentiated and effective to investigate different subtypes of *L. monocytogenes*. PCR-RFLP could be used as a screening step to identify certain *L. monocytogenes* subtypes before PFGE analysis is performed.

Although all of the isolates in this study were from the same processing facility and product line, diversity among the isolates, as documented in literature, was far less than expected. Only three clusters, and thus three different subtypes could be identified. All of the subtypes also contained isolates of both product and environmental origin and no correlations with specific sources could be established.

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References

Autio, T., Lundén, J., Fredriksson-Ahomaa, M., Björkroth, J., Sjöberg, A.M. & Korkeala, H. (2002). Similar *Listeria monocytogenes* profiles detected in several foods originating from different sources. *International Journal of Food Microbiology*, **77**, 83–90.

Borucki, M.K. & Call, D.R. (2003). *Listeria monocytogenes* serotype identification by PCR. *Journal of Clinical Microbiology*, **41**, 5537–5540.

Doumith, M., Buchrieser, C., Glaser, P., Jacquet, C. & Martin, P. (2004). Differentiation of the major *Listeria monocytogenes* serovars by Multiplex PCR. *Journal of Clinical Microbiology*, **42**, 3819–3822.

Farber, J.M. & Peterkin, P.I. (1991). *Listeria monocytogenes*, a food-borne pathogen. *Microbiological Reviews*, **55**, 476–511.

Fugett, E.B., Schoonmaker-Bopp, D., Dumas, N.B., Corby, J. & Wiedmann, M. (2007). Pulsed-field gel electrophoresis (PFGE) analysis of temporally matched *Listeria monocytogenes* isolates from human clinical cases, foods, ruminant farms, and urban and natural environments reveals source-associated as well as widely distributed PFGE types. *Journal of Clinical Microbiology*, **45**, 865–873.

Gerner-Smidt, P., Hise, K., Kincaid, J., Hunter, S., Rolando, S., Hyytia-Trees, E., Ribot, E.M. & Swaminathan, B. (2006). PulseNet USA: a five-year update. *Foodborne Pathogens and Disease*, **3**, 9–19.

Gottlieb, S.L., Newbern, E.C., Griffin, P.M., Graves, L.M., Hoekstra, R.M., Baker, N.L., Hunter, S.B., Holt, K.G., Ramsey, F., Head, M., Levine, P., Johnson, G., Schoonmaker-Bopp, D., Reddy, V., Kornstein, L., Gerwel, M., Nsubuga, J., Edwards, L., Stonecipher, S., Hurd, S., Austin, D., Jefferson, M.A., Young, S.D., Hise, K., Chernak, E.D. & Sobel, J. (2006). Multistate outbreak of listeriosis linked to turkey deli meat and subsequent changes in US regulatory ploicy. *Clinical Infectious Diseases*, **42**, 29–36.

Graves, L.M., Hunter, S.B., Ong, A.R., Schoonmaker-Bopp, D., Hise, H., Kornstein, L., DeWitt, W.E., Hayes, P.S., Dunne, E., Mead, P. & Swaminathan, B. (2005). Microbiological aspects of the investigation that traced the 1998 outbreak of listeriosis in the United States to contaminated hotdogs and establishment of molecular subtyping-based surveillance for *Listeria monocytogenes* in the PulseNet network. *Journal of Clinical Microbiology*, **43**, 2350–2355.

Hof, H. & Rocourt, J. (1992). Is any strain of *Listeria monocytogenes* detected in food a health risk? *International Journal of Food Microbiology*, **16**, 173–182.

Stellenbosch University http://scholar.sun.ac.za

Hyytiä-Trees, E.K., Cooper, K., Ribot, E.F. & Gerner-Smidt, P. (2007). Recent developments and future prospects in subtyping of foodborne bacterial pathogens. *Future Microbiology*, **2**, 175–185.

Jeffers, G.T., Bruce, J.L., McDonough, P.L., Scarlett, J., Boor, K.J. & Wiedmann, M. (2001). Comparative genetic characterization of *Listeria monocytogenes* isolates from human and animal listeriosis cases. *Microbiology*, **147**, 1095–1104.

Jones, D & Seeliger, H.P.R. (1983). Designation of a new type strain for *Listeria monocytogenes*. *International Journal of Systematic Bacteriology*, **33**, 429.

Kathariou, S. (2002). *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *Journal of Food Protection*, **65**, 1811–1829.

Liu, D. (2006). Identification, subtyping and virulence determination of *Listeria monocytogenes*, an important foodborne pathogen. *Journal of Medical Microbiology*, **55**, 645–659.

Mead, P. S., Dunne, E.F., Graves, L., Wiedmann, M., Patrick, M., Hunter, S., Salehi, E., Mostashari, F., Craig, A., Mshar, P., Bannerman, T., Sauders, B.D., Hayes, P., Dewitt, W., Sparling, P., Griffin, P., Morse, D., Slutsker, L. & Swaminathan, B. (2006). Nationwide outbreak of listeriosis due to contaminated meat. *Epidemiology and Infection*, **134**, 744–751.

Nucera, D., Lomonaco, S., Bianchi, D.M., Decastelli, L., Grassi, M.A., Bottero, M.T. & Civera, T. (2010). A five year surveillance report on PFGE types of *L. monocytogenes* isolated in Italy from food and food related environments. *International Journal of Food Microbiology*, **140**, 271–276.

Rasmussen, O.F., Skouboe, P., Dons, L., Rosen, L. & Olsen, J.E. (1995). *Listeria monocytogenes* exists in at least three evolutionary lines : evidence from flagellin, invasive associated protein and listeriolysin O genes. *Microbiology*, **141**, 2053–2061.

Rousseaux, S., Olier, M., Lemaître, J.P., Piveteau, P. & Guzzo, J. (2004). Use of PCR-restriction fragment length polymorphism of *inlA* for rapid screening of *Listeria monocytogenes* strains deficient in the ability to invade caco-2 cells. *Applied and Environmental Microbiology,* **70**, 2180–2185.

Sneath, P.H.A. & Sokal, R.R. (1973). Numerical Taxonomy: The principles and Practise of Numerical Classification. San Francisco: W.H. Freeman.

Tamburro, M., Ripabelli, G., Fanelli, I., Grasso, G.M. & Sammarco, M.L. (2010). Typing of *Listeria monocytogenes* strains isolated in Italy by *inl*A gene characterization and evaluation of a new cost-effective approach to antisera selection for serotyping. *Journal of Applied Microbiology*, **108**, 1602–1611.

Vogel, B.F., Huss, H.R., Ojeniyi, B., Hrens, P.A. & Gram, L. (2001). Elucidation of *Listeria monocytogenes* contamination routes in cold-smoked salmon processing plants detected by DNA-based typing methods. *Applied and Environmental Microbiology*, **67**, 2586–2595.

Wiedmann, M., Bruce, J.L., Keating, C., Johnson, A., McDonough, P.L. & Batt, C.A. (1997). Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. *Infection and Immunity*, **65**, 2707–2716.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Listeria monocytogenes is a bacterium that has only relatively recently emerged as a foodborne pathogen with the first confirmed case due to contaminated coleslaw, reported in 1981 (WHO, 2002; Montville & Matthews, 2008). Various food products, including meat, seafood, dairy products and vegetables have been identified as possible foods harbouring this bacterium (Meng & Doyle, 1997; Gugnani, 1999), with ready-to-eat (RTE) and minimally processed products being the most susceptible (WHO/FAO, 2004). Due to a lack of good agricultural practices and financial resources, vegetables are often the victim of microbiological contamination due to the use of contaminated organic fertilisers or untreated water (Francis *et al*., 1999).

Listeriosis, the disease caused by infection with *L. monocytogenes* is infective to all human population groups, however, the most susceptible groups include pregnant women, neonates, the elderly and immuno-compromised individuals such as those with HIV/Aids or tuberculosis (Vazquez-Boland *et al*., 2001). As the percentage of individuals contracting HIV/Aids and/or tuberculosis in developing countries are increasing, the risk of contracting listeriosis is of great concern. In South Africa there are limitations to the current surveillance system in place for reporting these cases and, therefore, the total number of listeriosis cases is unknown (WHO, 2007). The lack of knowledge in preserving and/or refrigeration of food products and a general increase in the consumption of RTE foods is an increasing concern for food producers.

Various conventional and molecular methods are available for the detection and identification of *L. monocytogenes*. Conventional methods are sensitive, inexpensive and provide results that are both qualitative and quantitative. Although this approach is the most common these methods are time-consuming and labour-intensive (De Boer & Beumer, 1999). They rely on phenotypic characteristics which are not only determined by the genotype, but also the environmental conditions that could lead to ambiguous results (Singleton & Sainsbury, 1978). Molecular methods are intrinsically more accurate by targeting unique genes for amplification and subsequent differentiation and, therefore, can be used to distinguish *L. monocytogenes* from other *Listeria* species. Molecular methods are also less effected by natural variation than conventional methods and offers high specificity, sensitivity and a rapid turnover which is essential for producers of minimally processed food products (Liu, 2006).

In this study both conventional and molecular methods were used to evaluate presumptive *L. monocytogenes* strains representing product and environmental samples from an avocado processing facility. Inconsistencies were obtained when the results from conventional methods, using the *Listeria*-specific agars Oxford and RAPID'*L.mono* were compared to the results obtained with the molecular methods using species-specific and multiplex polymerase chain reaction (PCR). Although both methods could sufficiently identify *Listeria* strains, identifying *L. monocytogenes* led to a discrepancy between the two methods. The species-specific and multiplex PCR, however, proved to be useful in detecting and identifying *L. monocytogenes* in a shorter period of time and with fewer inconsistencies than with the conventional methods.

Although *L. monocytogenes* is pathogenic at the species level, various strains exhibit varied virulence and pathogenic potential (Erdenlig *et al*., 2000; Gracieux *et al*., 2003; Liu *et al*., 2003b; Liu, 2004; Doumith *et al*., 2004). The difference between these strains is minimal as they all possess the same virulence gene cluster (LIPI-1), but may differ in the expression of these genes (Liu *et al*., 2003a). In this study, *L. monocytogenes* strains from the same avocado processing facility was subtyped by two molecular methods, PCR-restriction fragment length polymorphism (RFLP) and pulsed-field gel electrophoresis (PFGE). These two methods differ in the size of the DNA that is investigated, as well as the labour, time and financial resources needed to achieve results. Both of these techniques resulted in three clusters with a 98 % correlation between the results, with the PCR-RFLP being less expensive and obtaining results in a shorter period of time than PFGE. The PCR-RFLP assay used in this study may, therefore, be used as a screening tool to investigate the diversity of strains before being subjected to an epidemiological investigation.

Concluding remarks

The current challenges regarding *L. monocytogenes* in food products are the correct detection and identification of this bacterium in a relative short period of time. As food processors of exported products have to adhere to stringent laws regarding the presence of this bacterium, a rapid and reliable method is needed. Although this study indicated that the molecular methods were intrinsically more effective in identifying *L. monocytogenes*, RAPID*'L.mono* agar was sensitive and specific and could be used as a first-step to detect the incidence of *L. monocytogenes* contamination before further confirmation tests are carried out.

The results from this study indicated that three different *L. monocytogenes* strains are present in the avocado processing facility. No correlation could be identified between the clusters and the origin of the isolates and, therefore, the source of the *L. monocytogenes* contamination could not be identified. Further research is needed to determine the source of contamination, as well as to conclusively identify whether these strains express their virulent genes and subsequently pose a health risk to consumers.

References

De Boer, E. & Beumer, R.R. (1999). Methodology for detection and typing of foodborne microorganisms. *International Journal of Food Microbiology*, **50**, 119–130.

Doumith, M., Cazalet, C., Simoes, N., Frangeul, L., Jacquet, C., Kunst, F., Martin, P., Cossart, P., Glaser, P. & Buchrieser, C. (2004). New aspects regarding evolution and virulence of *Listeria monocytogenes* revealed by comparative genomics and DNA arrays. *Infection and Immunity*, **72**, 1072–1083.

Erdenlig, S., Ainsworth, A.J. & Austin, F.W. (2000). Pathogenicity and production of virulence factors by *Listeria monocytogenes* isolates from channel catfish. *Journal of Food Protection*, **63**, 613–619.

Francis, G.A., Thomas, C. & O'Beirne, D. (1999). The microbiological safety of minimally processed vegetables. *International Journal of Food Science & Technology,* **34**, 1–22.

Gracieux, P., Roche, S.M., Pardon, P. & Velge, P. (2003). Hypovirulent *Listeria monocytogenes* strains are less frequently recovered than virulent strains on PALCAM and Rapid'*L. mono* media. *International Journal of Food Microbiology*, **83**, 133–145.

Gugnani, H.C. (1999). Some emerging food and water borne pathogens. *Journal of Communicable Diseases*, **31**, 65–72.

Liu, D. (2004). Listeria monocytogenes: comparative interpretation of mouse virulence assay. *FEMS Microbiology Letters*, **233**, 159–164.

Liu, D. (2006). Identification, subtyping and virulence determination of *Listeria monocytogenes*, an important foodborne pathogen. *Journal of Medical Microbiology*, **55**, 645–659.

Liu, D., Ainsworth, A.J., Austin, F.W. & Lawrence, M. L. (2003a). Use of PCR primers derived from a putative transcriptional regulator gene for species-specific determination of *Listeria monocytogenes*. *International Journal of Food Microbiology*, **91**, 297–304.

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Liu, D., Austin, F.W., Ainsworth, A.J. & Lawrence, M. (2003b). Characterization of virulent and avirulent *Listeria monocytogenes* strains by PCR amplification of putative transcriptional regulator and internalin genes. *Journal of Medical Microbiology*, **52**, 1066–1070.

Meng, J. & Doyle, M.P. (1997). Emerging issues in microbiological food safety. *Annual Review of Nutrition*, **17**, 255–275.

Montville, T.J. & Matthews, K.R. (2008). *Listeria monocytogenes*. In: *Food Microbiology, An Introduction*, 2nd ed. Pp 173–187. Washington, DC: ASM Press.

Singleton, P. & Sainsbury, D. (1978). *Dictionary of microbiology*. p. 303. New York: John Wiley & Sons.

Vazquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehlan, J. & Kreft, J. (2001). *Listeria* pathogenesis and molecular virulence determinants. *Clinical Microbiology Reviews*, **14**, 584–640.

World Health Organisation (WHO) (2002). Foodborne diseases, emerging. Fact Sheet No. 124. [WWW document]. URL http://www.who.int/mediacentre/factsheets/fs124/en/. 21 October 2010.

World Health Organization/Food and Agriculture Organization (WHO/FAO) (2004). Risk assessment of *Listeria monocytogenes* in ready-to-eat foods, MRA Series No. 4 – 5. [WWW document]. URL http://www.who.int/foodsafety/publications/micro/mra_listeria/en/index.html. 23 March 2007.

World Health Organization (WHO) (2007). Global tuberculosis control: surveillance, planning, financing: WHO report 2007. Geneva, Switzerland: WHO Press.

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