The diversity and detection of

Listeria

in food and water in the Western Cape

by

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I declare that " The diversity and detection of *Listeria* in food and water in the Western Cape" is my own work and all the sources I have used or quoted have been indicated and acknowledged by means of complete references.

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Chapter 1

Introduction

Listeria is a gram positive non-sporing rod-shaped bacteria that is common in nature and a regular contaminant of a variety of raw and processed food (*Beuchat* and *Brackett*, 1990a; *Beuchat* et al., 1990). Currently, the genus comprises of seven species. Most research however, has concentrated on the human foodborne pathogen, *Listeria monocytogenes* which causes the disease listeriosis (*Jones*, 1990). Some epidemic foodborne outbreaks have indicated that there is a possible link between soil/water contamination and foodborne listeriosis (*Farber* and *Peterkin*, 1991). However, *Listeria* are easily inhibited by a variety of commonly used antibiotics such as tetracycline and bacteriocins produced naturally by starter cultures in a variety of processed food products (*Muriana*, 1988; *Facinelli* et al., 1991; *Giraffa*, 1995). The significance of the organism lies in the risk posed to the immunocompromised, the very young and the elderly and the high mortality rate associated with these groups. Added to this, there are many unresolved questions surrounding the minimum infective dose, the roles that various genes play in infection and the tolerance and adaptation of *Listeria* to adverse conditions (*Schlech*, 1988). Due to these factors, Europe currently imposes a "zero tolerance level" for all *Listeria* in any processed food products (*Czuprynski*, 1994). However, in South Africa listeriosis is not a reportable disease and some symptoms are "flu-like" which conceals the presence of *Listeria* (*Jones*, 1990). Therefore, the prevalence of this organism in the Cape Flats area may be underestimated. The implications of listeriosis are not only clinical but also have far reaching economical repercussions because of the "zero-tolerance" requirement currently enforced by the international community. This has led to a variety of detection and identification methods that are both rapid and sensitive (*Billie* et al., 1992; *Bubert* et al., 1992; *Herman* et al., 1995; *Jeršek* et al., 1996; *Beumer* et al., 1995). However, most detection methods (classical, phenotypical or genotypical) only concentrate on the detection and identification of *L.monocytogenes* while excluding all other *Listeria* or are optimised for a particular system (eg. meat, milk).

Thus, the aim of the work presented here was to determine the prevalence of *Listeria* in food and water in the Cape Flats area using classical (i.e. biochemical tests), phenotypical (whole-cell proteins) and genotypical methods (PCR of the *iap* gene and the conserved region of the 16SrRNA gene).

Chapter 2

Literature Review

2.1 Introduction

Murray et al. (1926) was accredited with the discovery of *Bacterium monocytogenes* that caused disease in laboratory animals. In 1927, *Pirie* discovered a similar organism in South African gerbils which was designated the name *Listerella hepatolitica* and later changed to the present name *Listeria monocytogenes*. Due to the initial discovery of this organism in animals, research before the 1980's was mainly focussed on the behaviour of this pathogen in laboratory, domesticated and wild animals and birds (*Fenlon*, 1985; *Hird*, 1986). Environmental habitats such as soil, water and plants were also investigated (*Blendon* and *Szatalowicz*, 1967; *Colburn* et al., 1990). Since the late 1970's with the implication of *L.monocytogenes* in various sporadic and epidemic outbreaks of listeriosis, *L. monocytogenes* has emerged as a foodborne pathogen (*Schlech* et al., 1983; *Farber* and *Peterkin*, 1991; *Salvat* et al., 1994). Although *L. monocytogenes* has mainly been linked to food, characteristics including its ability to withstand dehydration , grow in a wide pH range, form a natural part of the intestinal microflora of human and animal intestinal tracts and its ability to withstand unfavourable temperatures warrant greater research in natural environments such as soil and water (*Seeliger* and *Jones*, 1986; *Bayles* et al., 1996; *Patchett* et al., 1996; *Jones* et al., 1997). The severity of listeriosis in the elderly, neonates and immunocompromised individuals and the infectious dose not being known has resulted in a ' zero tolerance level' of *Listeria* spp. in food and water in Europe (*Czuprynski*, 1994). Little is known regarding the prevalence of *Listeria* species in soil and water and even less regarding the origin and factors affecting transfer to plant and animal-based foods. Factors that have made the detection of *Listeria* species in natural habitats difficult include the low ratio of *Listeria* to other organisms, the difficulty in isolating sufficient quantities of clean DNA template and the presence of inhibitors such as humic acids naturally found in soil and water. The advent of the molecular based methods such as Polymerase Chain Reaction (PCR) has resulted in the effect of these constraints being limited (*Steffan*

and *Atlas*, 1991).

2.2 Description of the genus *Listeria*

2.2.1 History of the genus *Listeria*

Murray et al.(1926) isolated an organism that caused mononucleosis in laboratory rabbits and named it *Bacterium monocytogenes* (*Farber* and *Peterkin*, 1991; *Seeliger* and *Jones*, 1986). In 1927 in an independent study, Pirie identified a gram positive bacteria that caused death in South African gerbils. Pirie named the isolate *Listerella hepatolytica* as a tribute to Lord Lister. However, after it was discovered that the two names characterized the same species, Pirie named the species *Listerella monocytogenes*. Since the genus name *Listerella* had already been used to describe mycetozoans, Pirie changed the genus name to *Listeria* (*Seeliger* and *Jones*, 1986). The only species that belonged to this genus was *Listeria monocytogenes*. In 1936 the link between disease in neonates and this pathogen was established (*Farber* and *Peterkin*, 1991). From the time of first being described to 1961, *L.monocytogenes* was the only species within this genus. After this *Listeria denitrificans* (1961), *Listeria grayi* (1966) and *Listeria murrayi* (1971) were recognised as belonging to the genus *Listeria*. In 1975, Ivanov described the bacterium that in 1984 became known as *Listeria ivanovii*. Soon after this a nonpathogenic strain, *Listeria innocua* was recognised, and in 1983 *Listeria welshimeri* and *Listeria seeligeri* were identified as species separate from *Listeria monocytogenes* (*Farber* and Peterkin, *1991*).

2.2.2 Current status of the genus

2.2.2.1. Physiological and morphological description of the genus

Listeria are gram positive, non-sporing, regular short (0.4-0.5 x 0.5-2μm) rods that appear coccoid in shape. They occur singly or in short chains and sometimes in long filaments. They are motile at 20-25 °C via peritrichous flagella. They are facultative anaerobes which grow optimally at temperatures between 30 and 37 °C. They are chemoorganotrophs which have a fermentative metabolism of glucose yielding mainly L(+)-lactate. The cells are catalase positive but cytochrome oxidase negative. Cells produce cytochromes. They are not acid fast and not encapsulated (*Seeliger* and *Jones*, 1986).

2.2.2.2 Current status of the species in the genus

Classification of *L.denitrificans*, *L.grayi* and *L.murrayi* has remained controversial. After extensive DNA-DNA hybridization, DNA base and numerical taxonomic studies, *Stuart* and *Welshimer* (1974) proposed that *L.denitrificans* be transferred to a new genus, *Jonesia* and renamed *Jonesia denitrificans*. DNA-DNA hybridization studies supported the proposal that *L.grayi* and *L.murrayi* be reclassified as *Murraya*. However rRNA cataloguing studies have shown that *L.murrayi* is closely related to *L.monocytogenes* (similarity coefficient of 0.73)(*Seeliger* and *Jones*, 1986). *Roucourt* et al. (1987), recognized that in the genus *Listeria* there are seven species that are closely related but they have distinct lines of origin. The one group consists of *L.monocytogenes*, *L.innocua*, *L.ivanovii*, *L.seeligeri* and *L.welshemeri*, while the other consists of *L.grayi* and *L.murrayi*. The reclassification of the later two species has, however, not been proposed to date.

Table 1. Differentiating between the characteristics for *Listeria* and *Jonesia* (adapted from *Farber* and *Peterkin*, 1991)

	Listeria	*Jonesia*
Cell Shape	Short, regular rods	slender, irregular rods
Cell arrangement	Singly, short chain sometimes long filaments	singly, branched and club-shaped sometimes filaments
Motility	20-25°C but not at 30- 37°C	25°C and 37°C
Gram reaction	Always gram positive	Normally gram positive when in coccoid form but older cultures decolourise easily
Voges-Proskauer	Positive	Negative

2.3 Pathogenicity of the genus *Listeria*

2.3.1 Listeriosis in humans

Although anyone can become infected with listeriosis, many people remain symptomless carriers (*Marth*, 1988). Nonetheless, individuals such as the elderly, newborn and those with underlying conditions that results in the suppression of T-cell mediated immunity (pregnancy, AIDS) are more likely to develop the disease (*Schlech*, 1988; *Jensen* et al., 1994). Since pregnant women have suppressed cell-mediated immunity, they are predisposed to invasive listeriosis. Usually the infection is manifested by flu-like symptoms of fever, chills, headache, backache and sometimes discoloured urine (*Marth*, 1988; *Gellin* and *Broome*, 1989) . However, they can also suffer mild symptoms or are asymptomatic carriers. Very rarely, is meningitis noted in pregnant women. However, listeriosis does not only affect the mother but also her fetus. The neonate can either become transplacentally infected or acquire infection during vaginal delivery (*Schlech*, 1988; *Schlech* et al., 1983). Since the infant has an immature immune system, listeriosis develops. Listeriosis in the fetus manifests as bacteraemia which results in shedding of *L.monocytogenes* in the urine. Discharge of infected urine, results in amniotic fluid being infected (amnionitis) which can lead to premature labour or septic abortion (*Marth*, 1988). Listeriosis is seldom fatal to the pregnant mother but in neonates fatality rates range between 3 to 50% in neonates born alive. Neonatal listeriosis is divided into two clinical forms: early and late onset. Early onset neonatal listeriosis usually occurs when infants are affected *in utero* when the mother has suffered from bacteraemia. On average, symptoms appear 1.5 days after birth. Usually, newborns suffer from lesions in the liver and placenta but sometimes also in the brain, adrenal gland, spleen, kidney, lungs and the gastrointestinal tract. In contrast, late onset neonatal listeriosis results in infants who underwent uncomplicated births. These infants are normally healthy at birth but develop symptoms on average 14 days after birth (*Gellin* and *Broome*, 1989). Usually these neonates develop meningitis. Nonperinatal listeriosis is an infection in cancer patients, transplant recipients, persons receiving immunosuppresive therapy, AIDS patients (*Jurado* et al., 1993) and the elderly (*Gellin* and *Broome*, 1989; *Lorber*, 1990). Other underlying disorders that result in susceptibility to listeriosis include diabetes, liver disease and renal disease. Patients with decreased gastric acid are also more susceptible. Nonetheless, 30 and 54% of adults and children respectively who develop listeriosis are not immunocompromised. Meningitis is the most

common manifestation of this disease in children and adults (*Gellin* and *Broome*, 1989).

2.3.2 Mode of action and genes involved in pathogenicity

For *L.monocytogenes* to function successfully as an intracellular pathogen, the following criteria must be fulfilled : the organism must be present in certain numbers , the organism must attach and enter the epithelial cells. Once inside the host cell the organism should avoid the hosts defence system, multiply and protect itself by producing toxins (*Czuprynski*, 1994).

The majority of virulence genes of *L.monocytogenes* are concentrated in a 10kb fragment on the chromosome. These genes include *hly, plcA, plcB, mpl, actA,inlA, prfA*. The only gene located outside this fragment is the *imt* gene. Genes such as *iap, sod, cat* and *dth* have been associated with pathogenicity but the role they play has not been established. Invasion of the epithelial cells requires a 80kDa protein known as internalin (coded for by the *inlA* gene) and possibly a 47.5kDa protein, the invasion associated protein *(iap* gene)(*Datta*, 1994; *Wuenscher* et al., 1993) (Fig. 1.).
The host defence system becomes activated and the invasive *Listeria monocytogenes* are taken up in the phagolysosome within 30 minutes. The phagolysosome contains bactericidal enzymes, therefore the *hly* gene is activated and 58kDa SH-activated haemolysin (named listeriolysin O) is secreted which dissolves the phagolysosome membranes to release the bacteria into the cytoplasm of the host cell. Within 90 minutes, cell division takes place and the bacteria are covered with actin filaments. Actin polymerization involves a 90kDa surface protein produced by the virulence gene, *actA*. The actin filaments are then organised in a comet-like shape (*imt* gene) with the tail turned inwards. This comet-like shape facilitates both intra and intercellular movement, since bacteria without the *imt* gene are avirulent, thus proving that intercellular movement is necessary for virulence. The bacterium with the actin tail protrudes from the host cell, is immediately enveloped by macrophages and enclosed by two host membranes. This double membrane is dissolved by a lecithinase (produced by *plcB)* and possibly also listeriolysin O. This results in the bacteria being released and the infection cycle is repeated (*Datta*, 1994).

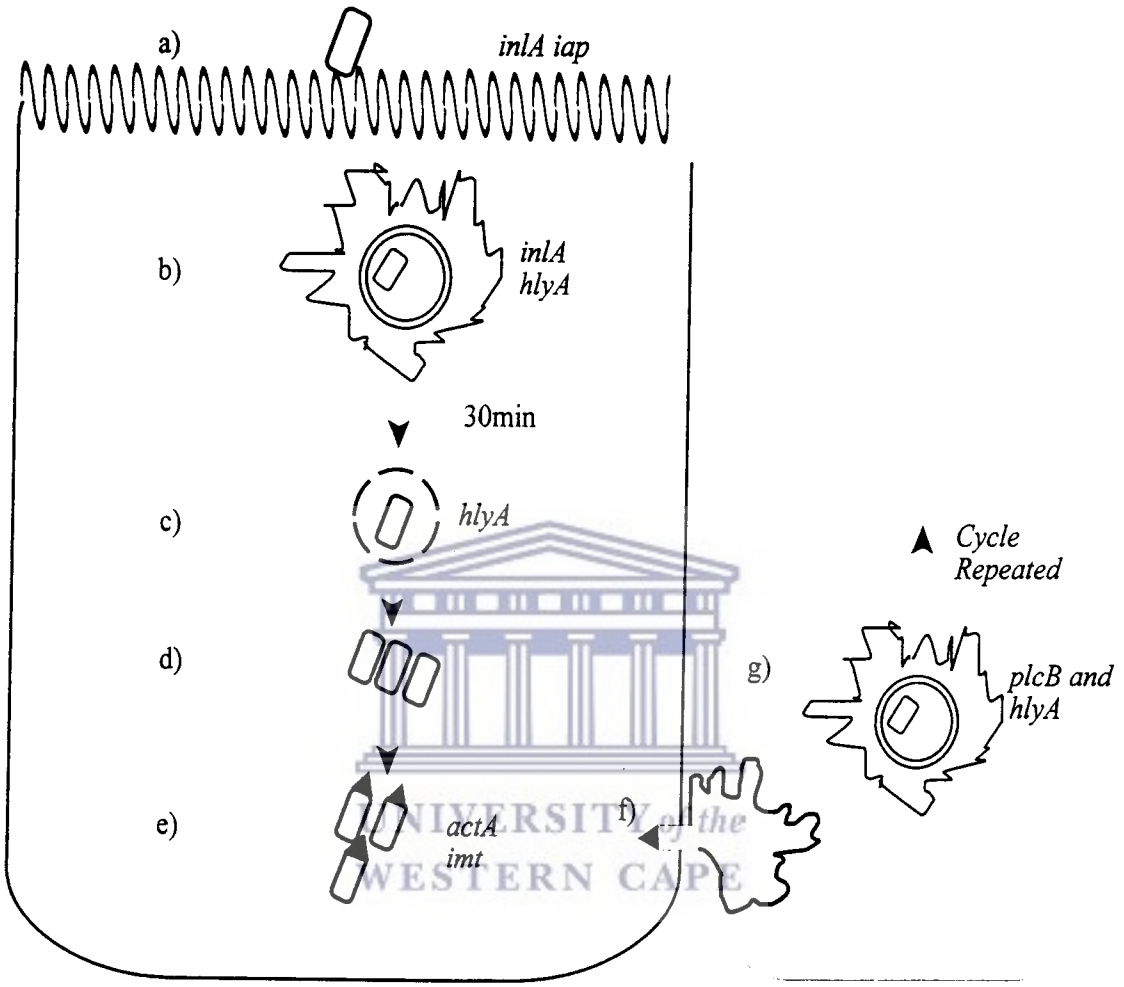

Fig. 1 The infection cycle of *L. monocytogenes*.

a) Intestinal epithelial cells invaded through the production of internalin (*inlA*) and invasion associated protein (*iap*).
b) Host defence activated and *L. monocytogenes* taken up into the phagosome.
c) Listeriolysin O (*hlyA*) together with internalin form pores in the host cell resulting in the escape of *L. monocytogenes*.
d) Multiplication of *L. monocytogenes*.
e) Surface proteins produced by *actA* result in actin polymerization, while the comet-shaped actin tail is as a result of protein production by *imt* gene.
f) The actin tail activates macrophages to engulf *L. monocytogenes* and a double membrane vesicle is formed.
g) Lecithinase (*plcB*) possibly with listeriolysin O dissolve the double membrane which results in the release of *L. monocytogenes* and a repetition of the infection cycle in a new host cell..

2.3.3. Epidemic and sporadic outbreaks linked to *Listeria*

2.3.3.1. Foodborne epidemic outbreaks of listeriosis

The means of transmission of *L.monocytogenes* in both sporadic and epidemic cases was still unknown till recently although cutaneous listeriosis in vets delivering infected calves has been demonstrated (*Schlech* et al., 1983). However, in 1981 in the Maritime Provinces in Canada (Table 2) an epidemic outbreak involving 41 cases (34 perinatal and 7 adults) occurred. Of the perinatal cases; 5 cases were spontaneously aborted, 4 cases stillbirths, 23 cases of babies born alive and 2 cases of alive babies who were healthy. Of the 23 babies born alive, 6 later died despite antibiotic therapy and supportive care. The source of illness was later traced to consumption of contaminated coleslaw. The coleslaw which was contaminated with *L.monocytogenes* serotype 4b was obtained from the refrigerator of the one patient. This was the epidemic strain and also the one isolated from the patient's blood. The coleslaw was prepared by a regional manufacturer who obtained his carrots and cabbage from wholesalers as well as local farmers. Evaluation of the processing activities revealed no internal contamination. However, after cold enrichment of two unopened supermarket coleslaw samples, *L.monocytogenes* serotype 4b was isolated (*Farber* and *Peterkin*, 1991; *Schlech* et al., 1983).

In an outbreak in 1979 involving *L.monocytogenes* serotype 4b, 20 cases of listeriosis occurred in adult patients, 50% were immunosuppressed because of chemotherapy or steroid treatment. The mortality rate was 15%. The implicated foods were raw vegetables such as lettuce, celery and tomatoes (*Farber* and *Peterkin*, 1991).

In Massachusetts in 1983, an outbreak of listeriosis involving pasteurised milk resulted in 49 adult and perinatal cases, of which 29% (14) died. On investigation, it was found that the milk came from a farm where bovine listeriosis had occurred. However, there was nothing inappropriate about the pasteurisation process, so it was suggested that the intracellular location of the *L.monocytogenes* resulted in greater heat resistance (*Farber* and *Peterkin*, 1991).

In California in 1985, 142 cases (93 perinatal and 49 adults) of listeriosis was reported. This outbreak resulted in a total of 48 deaths. Mexican-style soft cheese was connected to the epidemic strain *L.monocytogenes* serotype 4b. Environmental samples were positive for the epidemic strain and 11% of the test cheese samples were positive for phosphatase. Although none of the raw milk from the various farms was contaminated, it was revealed that the rate of delivery was too high for the pasteuriser, resulting in incomplete pasteurization. The cheese plant was closed down and all Mexican-style cheese recalled (*Farber* and *Peterkin*, 1991).

In July 1986 Polar Bar ice-cream bars were withdrawn from the market after *L.monocytogenes* was detected during routine sampling. Approximately 330 people were exposed of which 42 were pregnant women. No significant symptoms developed in the women or their fetuses suggesting that exposure does not necessarily lead to disease (*Gellin* and *Broome*, 1989).

Another outbreak occurred in the Western part of Switzerland involving Vacherin Mont d'or soft cheese. During 1983 to 1987, 122 cases were reported and in 85% of the cases the epidemic strain was isolated. This outbreak resulted in 31 fatalities and the recall of the cheese in November 1988 (*Billie*, 1990).

Table 2. Epidemic foodborne outbreaks due to *L.monocytogenes* (Adapted from *Farber* and *Peterkin*, 1991; *Gellin* and *Broome*; *Salvat* et al., 1994)

Location	Year	Source	No. cases(fatalities)
Boston	1979	Raw salad vegetables	20 (5)
Maritime Provinces	1981	Coleslaw	41 (17)
Massachusetts	1983	Pasteurized milk	49 (14)
California	1985	Mexican-style cheese	142 (48)
Switzerland	1983-1987	Raw milk, cheese	122 (31)
France	1992	Pork tongue	279 (85)

Usually epidemic listeriosis is linked to a particular food or food type. However, epidemic outbreaks have occurred where the contaminated food product has only been epidemiologically implicated. Examples of these are Philadelphia (1986-1987) where ice-cream and salami were implicated, the United Kingkom

(1987-1989) where páte was implicated, New Zealand (1980) and Conneticut (1989) where seafood was implicated (*McLaughlin* et al., 1991; *Farber* and *Peterkin*, 1991).

2.3.3.2 Sporadic outbreaks of listeriosis

Most cases of listeriosis occur sporadically and not in identifiable clusters as in the case of epidemic listeriosis. *Farber* and *Peterkin* (1991) have reviewed 17 cases of point-source illness in which a variety of foods have been implicated in illness amongst both healthy and immunocompromised idividuals ranging in age from 24-66 years.

2.4 Methods for the detection and identification of *Listeria*

2.4.1 Phenotypical identification of the genus

2.4.1.1 Classical cultural methods for the isolation of *Listeria*

Earlier methods such as the cold enrichment method were developed for clinical or veterinary use. However, the correlation between *Listeria* and foodborne disease has resulted in the development of improved methods for detection of *Listeria* in natural and food-based environments. The advantages of detection in clinical samples is that there are usually large numbers of organisms, growing in almost pure culture under ideal conditions. In contrast, detection of *Listeria* in food-based samples is more difficult since these pathogens occur in low numbers relative to competitive bacteria and they may be sublethally stressed because of food processing conditions. Thus, to be able to detect *Listeria* in food samples the enrichment and isolation method must be both selective (isolate *Listeria* from background flora) and sensitive (able to isolate low numbers of *Listeria* or sublethally stressed cells). The classical analysis of foodborne pathogens like *Listeria* entails an enrichment, isolation and confirmation step (*Buchanan*, 1990).

The enrichment phase is used to amplify the number of target microbes while suppressing the growth of competitive bacteria in the sample. Initially, the cold enrichment method developed by *Gray* et al. (1948) was used extensively to isolate the psychrophyllic *Listeria* from background flora. The disadvantage of this system was that it required prolonged incubation since the generation time of *Listeria* at 4°C is 1.5 days. Henry's transillumination method (Fig. 2.) for the isolation of *Listeria* on colourless agar was the initial method used for the identification of *Listeria* from any colourless agar (*Lovett*, 1988).

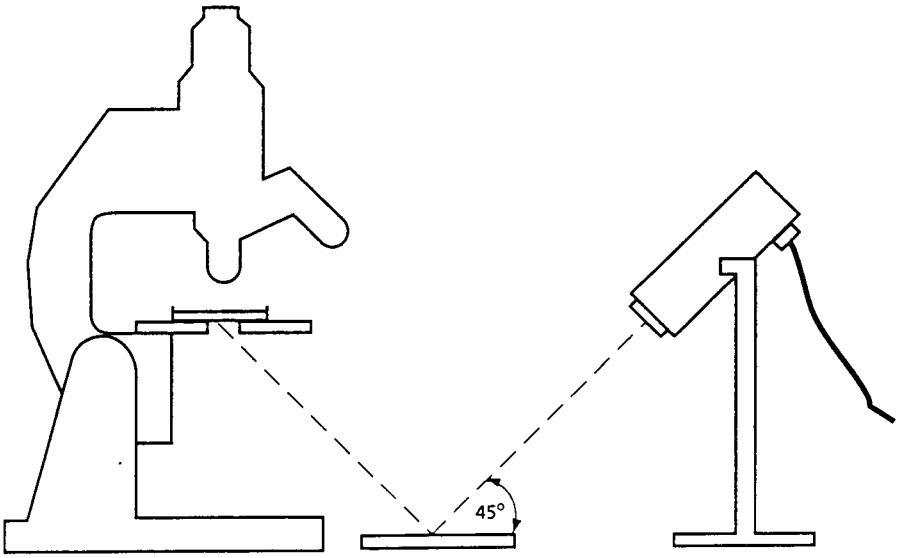

Fig 2. Henry's apparatus for viewing colonies of *Listeria monocytogenes* with obliquely reflected light (*Buchanan*, 1990).

Presumptive *Listeria* colonies were blue to blue-grey in colour when illuminated with obliquely reflected light (Fig.2). However, the disadvantages of this method is that it is difficult to use effectively, subjective to observations and cannot be used for quantitative analysis. McBride's Listeria media (MLA) was the media originally used for the isolation of *Listeria* from mixed cultures and forms the basis for all media that were developed later. The selective components of this media were phenylethanol, lithium chloride, glycine and blood. Both phenylethanol and lithium chloride were found to be inhibitory to gram negative organisms (*Cassidy* and *Brackett*, 1989). The blood was included in the media to identify hemolytic *Listeria* (*Buchanan*, 1990). However, the drawback of this media was that resistant staphylococci and streptococci outgrew all other bacteria. To overcome these shortcomings, *Lovett* (1988) eliminated the blood, added cycloheximide to suppress the growth of moulds and replaced the glycine (since it was found to be inhibitory to the recovery of *L.monocytogenes*) with glycine anhydride (*Cassidy* and *Brackett*, 1989). This media became known as Modified McBride's Agar (MMA). *Lee* and *McCLain* (1986) combined the advantageous components of MLA with Baird-Parker medium which resulted in LiCl Phenylethanol Moxalactam Agar (LPMA). Similar in compostion to MMA, this medium , also contained the wide spectrum β-lactam antibiotic,

is usually selective, occasionally, resistant enterococci, staphylococci and *Kurthia* still grew on this modified media. Another disadvantage was that not all *Listeria* colonies that grew on this media were the characteristic blue colour when observed under obliquely reflected light. Despierres Listeria Agar (DLA) used the selective effects of acriflavine, nalidixic acid, polymycin B and methylene blue together with the sugar rhamnose to promote the growth of *L.monocytogenes*. As with all the previous methods, differentiation was based on the characteristic colony colour under obliquely reflected light. In comparing RAMPANY and LPMA, *Ralovich* (1989) found that LPMA was the most sensitive and selective plating media for isolation of *L.monocytogenes* from sausages and milk. It was concluded from these results that LPMA should be more widely used. The selective agents and antibiotics commonly used are listed in Table 3.

Later, modified media from which *L.monocytogenes* could be isolated because of indicators in the media instead of using obliquely reflected light for differentiation, were developed. These included , PALCAM, Oxford and modified Oxford agars. Ultimately the type of media used depends on the injury caused to the bacteria by environmental or processing stresses, the source of the isolate and the contamination caused by background flora (*Buchanan*, 1990).

Table 3. Selective agents and antibiotics in commonly used media for the isolation of *Listeria* from mixed cultures (Adapted from *Buchanan*, 1990; *Ralovich*, 1989)

Medium	Antibiotic [a]	Selective agents [b]
McBride's Listeria Agar (MLA)	None	PE, LC, G
Modified Mcbride's Agar (MMA)	Cyclo	PE, LC, GA
Litium Chloride Phenylethanol Moxalactam (LPMA)	Mox	GA, PE, LC
Modified Vogel Johnson Agar (MVJA)	Mox, Nal, Bac	GA, LC, PT
RAPAMY	Mox, Nal	A, PE
PALCAM	Cz, Pol	A, LC, E
Oxford Agar	Ctt, Fos, Col	A, LC, E
Modified Oxford Agar	Mox, Col	LC, E

[a] Abbreviations: Cyclo, cycloheximide; Mox, moxalactam; Nal, nalidixic acid; Bac, bacitracin; Cz, ceftazidime; Pol, polymyxin B; Ctt, cefotetan; Fos, fosfomycin; Col, colistin.[b] GA, glycine anhydride; PE, phenylethanol; LC, lithium chloride; PT, potassium tellurite; E, aesculin; Ktc, potassium thiocynate; G, Glycine; A, acriflavine.

2.4.1.2 Conventional methods for identification

Traditionally, the identification of isolates requires colony and cellular morphology, growth at 37°C, relationships to oxygen and catalase production, motility and a variety of biochemical tests listed in the Table 4 below.

Table 4. Biochemical characteristics for the identification *Listeria* (Adapted from *Seeliger* and *Jones*, 1986)

Characteristics	**Reaction**
Motility (at 20-25 °C)	+
Oxygen requirement	facultative
Growth at 35 °C	+
Catalase activity	+
Hydrogen sulphide production	-
Acid from glucose	+
Methyl red reaction	+
Voges-Proskauer reaction	+
Indole production	-
Citrate utilisation	-
Urease activity	-

The biochemical tests given in the table above are used to identify organisms belonging to the genus *Listeria*. For biochemical characterisation to the species level, further tests have to be performed (Table 5) which includes the CAMP test. The CAMP test is the process whereby microbes growing adjacent to each other on the surface of a blood agar plate, produce diffusible exosubstances, which cause the synergistic lysis of red blood cells. This test is essential in the identification of *Listeria* species and also an indicator of pathogenicity. The CAMP test distinguishes between the haemolytic and pathogenic *L. monocytogenes* and *L. innocua* (non-haemolytic and non-pathogenic). The synergistic reaction between *Staphylococcus aureus* and *L. monocytogenes* is thought to be due to the production of a sphingomyelinase C (*S.aureus*) and a phosphatidylinositol-specific phospholipase C and a phosphatidylcholine specific phospholipase C (*L.monocytogenes*) while the reaction with *Rhodococcus equi* is due to the reaction between listeriolysin O (*L.monocytogenes*) and cholesterol oxidase (*R.equi*) (*Garayzàbal* et al., 1990; *McKeller*, 1994).

Table 5. Biochemical characterisation of *Listeria* to the species level (Adapted from *Lovett*, 1988)

	L.monocytogenes	*L.innocua*	*L.ivanovii*	*L.seeligeri*	*L.welshimeri*	*L.grayi*	*L.murrayi*
β-haemolysis	+	-	+	+	-	-	-
Catalase	+	+	+	+	+	+	+
Nitrate reduction	-	-	-	-	-	-	+
Urea hydrolysis	-	-	-	-	-	-	-
Methyl red	+	+	+	+	+	+	+
Voges Proskauer	+	+	+	+	+	+	+
TSIA [a]	A/A [b]	A/A [b]	A/A [b]	A/A [b]	A/A [b]	A/A [b]	A/A [b]
Motility	+	+	+	+	+	+	+
Dextrose	+	+	+	+	+	+	+
Aesculin	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Mannitol	-	-	-	-	-	+	+
Rhamnose	+	V [c]	-	-	V [c]	-	V [c]
Xylose	-	-	+	+	+	-	-

[a] TSIA Triple Sugar Iron Agar, [b]Acid slant/ acid butt, [c]Variable

2.4.1.3 Ident ification by means of whole cell proteins

Protein profiles for various organisms have been used as a tool to differentiate bacteria on both the genus (*Vogel* et. al., 1997) and species level (*Costas* et al., 1994). However, in the case of *Listeria*, the confirmation of the genus with reference to their whole-cell proteins is difficult, due to subgrouping or clustering of one species close to another. *Kämpfer* et al. (1994) found that two distinct clusters were observed at a correlation level of 0.15 (15%); one cluster contained *L.grayi* while the other contained all other *Listeria* species. At a correlation level of 0.62 (62%), *L.welshemeri*, *L.ivanovii* and *L.seeligeri* clustered in one subgroup while *L.monocytogenes* and *L.innocua* clustered in the other subgroup. In addition at a correlation level of 0.70 (70%), the first subgroup grouped into separate clusters. *L.monocytogenes* also grouped into two separate subgroups. However, *L.monocytogenes* SLCC 53 remained unclustered but its protein profiles were similar to *L.innocua*. Similarly, most *L.innocua* clustered together except SLCC 8790 , which remained unclustered but whose profiles was similar to *L.monocytogenes*. *Gormon* and *Phan-Thanh* (1995) used two-dimensional protein mapping for identification of *Listeria* . They found this procedure to be more sensitive than one dimensional whole cell protein profiles. However, this procedure, although being more sensitive is more time-consuming, labour intensive and complicated than either one-dimensional SDS-PAGE or some of the other genotypical methods.

2.4.1.4 Identification by Enzyme-linked immunoassay (ELISA)

ELISA (Enzyme-linked immunoassay) can be used in conjunction with culture methods to confirm the identification of an organism. *Loiseau* et al. (1995) developed and characterised monoclonal antibodies that detects two heat stable intracellular genus specific antigens (38 and 41KDa) irrespective of serotype. *Kerr* et al.(1990) compared the results of conventional culturing methods and ELISA for the detection of *Listeria* in chicken. They found that the ELISA method yielded no false positive but only detected 24 of the 29 culture positive isolates.

2.4.1.5 Methods for the typing of *Listeria*

2.4.1.5.1 Serotyping

Initially, *Listeria* were typed on the basis of their agglutination reaction with O and H antigens and divided into four groups according to *Paterson* (1940). Since then, this scheme has been refined by Seeliger and Donker-Voet and consists of 16 serovars: 1/2a, 1/2b, 1/2c, 3a,3b,3c, 4a,4ab, 4b, 4c, 4d, 4e, 5, 7, 6a and 6b (*Seeliger* and *Jones*, 1986). One important limitation of this system is that it cannot be used to find the source of contamination since various human and animal strains belong to a limited number of serovars. Although antisera are available commercially, they only identify strains belonging to serovars 1 and 4.

2.4.1.5.2 Phage Typing

Loessner and *Busse* (1990) developed a bacteriophage system for typing *Listeria* since all species contain lysogenic phages. They found that the overall typability was 84.5%. However, the limitations of this system were that some *Listeria* species (*L.murrayi* and *L.grayi*) were resistant to lysis while *L.monocytogenes* strains belonging to serotype 3 were resistant to phage typing.

2.4.1.5.3 Other typing methods

The shortcomings of older methods such as serotyping and phage typing has led to several DNA-based fingerprinting techniques including ribotyping (*Arimi* et al., 1996; *Graves* et al., 1994), multilocus enzyme electrophoresis (*Bibb* et al., 1990) and random amplified polymorphic DNA (*Kerr* et al., 1995; *Niederhauser* et al., 1994; *Farber* and *Addison*, 1994; *Boerlin* et al., 1995; *Black* et al., 1995; *Wagner* et al., 1996).

2.4.2 Genotypical detection and identification of the genus

2.4.2.1 The use of probes targeting virulence genes

The use of virulence genes for the detection of *Listeria* has been widely used. *Chenevert* et al. (1989) found that probes containing part of the listeriolysin O gene hybridized to all *L.monocytogenes strains*

even those that are weakly or non-haemolytic. Using the 3.1kb fragment containing the *hylA* gene as a probe *Datta* et al. (1988) found that of the 166 strains tested (150 *Listeria* strains and 16 non-*Listeria*), this probe only hybridised to *L.monocytogenes*. *Kim* et al. (1991a; 1991b) using a 321bp internal fragment of another virulence gene, *msp* (major secreted polypeptide) found that the probe only hybridised to *L.monocytogenes* and not to other related species or genera.

2.4.2.2 The polymerase chain reaction (PCR)

2.4.2.2.1 General considerations for the PCR

The polymerase chain reaction was invented in 1983 by Kary Mullis and has been proclaimed as one of the most pivotal discoveries of this decade (*Ehrlich* and *Sirko*, 1994). The polymerase chain reaction permits the *in vitro* replication of defined DNA sequences, whereby gene segments can be multiplied. This amplification process involves the following steps: a) denaturisation in which double-stranded DNA unwinds into two single strands ; b) annealing of the primers to the target DNA; and c) elongation in which a new DNA strand is extended from the primers by the action of DNA polymerases. Since the PCR is a cascade of interlinking events, each step cannot be seen in isolation but has an effect on one or more other steps resulting in an unsuccessful or successful outcome. Parameters that affect the outcome of the PCR can be divided into two categories namely, internal and external parameters. Internal parameters include the different steps (denaturation, annealing and elongation) and the different components (primers, buffers, magnesium, Taq polymerase, enzyme stabilizers, DNA template), whereas external parameters include natural inhibitors, environmental inhibitors , sample used and background competition (*Steffan* and *Atlas*, 1991).

2.4.2.2.2 Steps of the PCR

The PCR cycles begin with a denaturisation step in which both template strands are separated to facilitate the binding of primers to the template. Typically, target sequences that are equal to or less than 1kb, require a denaturisation temperature of 94°C for 1min. The denaturisation time and temperature are important factors for the complete denaturisation of genomic DNA rich in GC sites. The denaturation of

these templates can be enhanced by the addition of additives and cosolvents (*Ehrlich* and *Sirko*, 1994). These substances include formamide, tetramethylammonium chloride and glycerol. Formamide when used in low concentrations in PCR cocktails are thought to help amplify GC rich targets by aiding strand separation. In contrast, excessive amounts of magnesium, result in the stabilization of double-stranded DNA and prevent complete DNA denaturation. Likewise, PCR buffers also play a crucial role in the denaturation process. Buffers provide an ionic environment in which all components of the PCR functions. Sodium chloride buffers increase the specificity in GC rich areas by providing more complete denaturation.

The annealing temperature is the temperature at which the primers bind to complementary sequences on the target DNA. Base composition, length and composition of the primers all play a pivotal role in determining the optimum primer annealing temperature. The annealing temperature is generally 5 °C below the true melting temperature of the primers. Typically, annealing temperatures in the 55 to 72 °C range yield the best results. Specificity of amplification is enhanced by the annealing temperature being at the lowest dissociation temperature (T_d) of either primer. The dissociation temperature is the temperature at which one - half of the primers are annealed to their target sequences and approximately calculated by the equation: $T_d = 4(G+C) + 2(A+T)$ (*Steffan* and *Atlas*, 1991). As genetic intricacy of a sample increases, the opportunity for nonspecific annealing also increases. This results in products that either have bands of a higher or lower molecular weight than those expected and which cannot bind to internal probes. Whereas high concentrations of magnesium ions affect denaturation, lower concentrations affect annealing of the primer set. Low concentrations result in stabilizing primer annealing to incorrect target sites, which results in lower specificity and the production of large amounts of non-target PCR products (*Ehrlich* and *Sirko*, 1994).

Primer extension is usually at 72 °C as this is the optimal activity temperature for the enzyme Taq polymerase. The extension depends on the temperature as well as the sequence and concentration of the target DNA. Additionally, if the concentration of magnesium ions is too low, the extension reaction will

be impaired as magnesium is a co-factor for most DNA polymerases (*Steffan* and *Atlas*, 1991).

2.4.2.2.3 Components of PCR

2.4.2.2.3.1 Deoxynucleotide

Atlas and *Steffan* (1991) recommend a concentration range between 20 and 200μM for maximum specificity and fidelity. The concentration of the individual nucleotides has to be equivalent to prevent misincorporation errors.

2.4.2.2.3.2 Taq polymerase

Initially, the Klenow fragment of DNA PolI was used but had to be replenished after every cycle since it was not heat stable. However, the isolation of heat stable DNA polymerases from thermophilic bacteria such as *Thermus aquaticus*, eliminated the need to continuously replenish the enzyme (*Felice* and *Alshinawi*, 1996). The concentration of Taq polymerase should be limited to a range between 1.0-2.5U per 100μl. Polymerase concentrations in excess of these concentrations will result in amplification of non-target background products. Whereas, too low a concentration can result in insufficient target DNA being produced. The optimum temperature of activity for Taq polymerase is around 70°C. Although it is not inactivated by short incubation periods where PCR product DNA will denature (usually 90-95°C), the half-life of Taq is 40 min at 95°C. Too many cycles at this temperature can lead to and increase in the yield of nonspecific products and an unnecessary loss of enzyme activity, whereas too few cycles will result in a low product yield (*Steffan* and *Atlas*, 1991).

2.4.2.2.3.3 Primers

Primer concentrations between 0.1-0.5μM are suggested. Primer concentrations in excess of the suggested range may encourage non-target product formation and also increase the production of primer-dimers (*Steffan* and *Atlas*, 1991). However, if the primer-template ratio is too low then the newly synthesized strands will renature to each other (*Ehrlich* and *Sirko*, 1994). Both non-specific products and primer-dimers result in a lower yield of the desired product. Primers should be between 18-20 base

pairs to be unique in both the complex eukaryote genome and the simpler prokaryotic genome and have a G + C composition of 50-60%. The dissociation temperature of the two primers should be roughly the same. Primers should not be complementary at the 3' end as this, will lead to self-annealing. Primers should have a T nucleotide at the 3' end as there is a greater chance of mismatches occurring with 3 or more Cs or Gs at the 3' end. Palindromic sequences within the primers should be avoided. Likewise, secondary structures of both target DNA and primers should be eliminated. If possible primer sequences should be tested against a database to prevent apparent errors in design (*Steffan* and *Atlas*, 1991; *Ehlrich* and *Sirko*, 1994).

2.4.2.2.4 Contamination

Usually amplified products contaminate unamplified PCR mixes. To prevent this all equipment used before and after amplification should be separated. To determine whether contamination has taken place, negative controls or water blanks should be included in each experiment (*Steffan* and *Atlas*, 1991).

2.4.2.2.5 External parameters

Inhibitory substances are found in many materials of interest (eg. blood, cheese, milk, water and soil) (*Kreader*, 1996). The reliability of the PCR is dependant on both the isolation of the bacterial target DNA and inhibition caused by samples (*Bickley* et al., 1996). *Dickinson* et al.(1995) reported that the presence of high background non-target DNA did not inhibit but in fact enhanced the PCR by acting as a target DNA carrier during nucleic acid precipitation. Clinical, food and environmental samples have all been shown to contain both natural and environmental PCR inhibitors. In blood, heme as well as its derivatives are known to block the PCR process. In addition, the breakdown products of heme (i.e. bilirubin and bile salts) which are found in faeces-contaminated samples also hinder the PCR. In addition to bilirubin and bile salts, the faeces of herbivores also contain large quantities of degraded plant matter which results in the formation of humic acid, fulvic acid and tannic acids (produced during the decomposition of plant matter) (*Kreader*, 1996) . These phenolic acids are therefore ubiquitous in soil and water and can possibly contaminate other samples including food. All three substances are phenolic

compounds that bind proteins (Taq polymerase) by forming hydrogen bonds with peptide bond oxygens which inactivates the Taq polymerase. *Kreader* (1996) found that bovine serum albumin (BSA) and T4 gene 32 protein (gp32) at concentrations of 400ng and 150 ng per μl respectively relieved interference caused by hemin, fulvic acids, humic acids, tannic acids, extracts from faeces, marine and fresh water. In addition, BSA also bound other substances including anions (due to high content of lysine) and lipids (via hydrophobic forces). Besides being contaminated by the environment, food contains natural PCR inhibitors. Inhibition of PCR by food compounds is usually overcome by diluting the sample, isolating the bacteria and /or extracting the DNA. Salmon contains phenolics, cresols and alderhydes which are all inhibitors of PCR (*Simon* et al., 1995), whereas the enzyme proteinase found in milk degrades Taq polymerase thus, inhibiting PCR (*Bickley* et al., 1996). Furthermore, they found that calcium ions in milk compete with magnesium ions for binding to Taq polymerase. Since magnesium is a cofactor for Taq, the binding of calcium interferes with this interaction resulting in the PCR being hindered. *Bickley* et al. (1996) also proved that the lipid content of milk did not inhibit the PCR but rather that there were other interfering substances.

Table 6. Summary of literature describing the various PCR for detection of *Listeria monocytogenes*

Sample tested	**Target DNA**	**Reference**
Type strains of *Listeria* cultures and other genera	Listeriolysin O gene and 16S rRNA gene	*Border* et al., 1990
L.monocytogenes inoculated into water, homogenized whole milk and cerebrospinal fluid	606-bp segment of *hlyA* gene encoding the listeriolysin O protein	*Bessesen* et al., 1990
ATCC *L.monocytogenes* strains, clinical isolates and cultures from LCDC	174-bp segment of listeriolysin O (*hlyA*) gene	*Deneer* and *Boychuk*, 1991
Human, meat, milk and the environment	200-bp segment located downstream of *hlyA* gene	*Rossen* et al., 1991
Cheese	Dth 18-gene	*Wernars* et al., 1991
L.monocytogenes Sv/2a EGD (University of Ulm,Germany) and other *Listeria* (Wurzburg,Germany)	1.5 kb fragment of *iap* gene (encoding)	*Bubert* et al., 1992
UHT, pasteurized or whole milk samples spiked with ATCC *L.monocytogenes* strain	*hlyA* promoter region (505bp)	*Starbuck* et al.,1992
Chicken and soft cheese	*hyl A* gene spanning a conserved Hind III site	*Fitter* et al., 1992
ATCC, SLCC and NCTC *Listeria* type strains and naturally contaminated samples	Multiplex PCR for *hlyA* and *iap* genes yielding a 234 and 131-bp products	*Niederhauser* et al.,1992
Blue Castello soft cheese spiked with *L.monocytogenes* 167vet (SMRI, Sweden)	553 and 275-bp fragment of 16SrRNA gene	*Lantz* et al., 1994
L.monocytogenes NCTC7973	*hly* gene	*Masters* et al., 1994
Camembert cheese,chicken and coleslaw inoculated with type strains from different sources	1052bp product of *prfA* gene	*Dickinson* et al., 1995
Food isolates and NCTC type strains	448bp internal fragment of *flaA* gene (flagellin protein)	*Gray* and *Kroll*, 1995
Soft cheese and minced meat artificially contaminated with *L.monocytogenes* EGD	626-bp fragment	*Makino* et al., 1995

Sample	Target DNA	Reference
Isolates from food, humans, animals and the environment in Sweden	2916 bp fragment including *inlA* encoding internalin and *inlB* (presumptive signal peptide)	*Ericsson* et al., 1995
Salmon seeded with *Listeria* NCTC strains and *L.seeligeri* isolated from naturally contaminated salmon	*prfA* gene (regulation of listeriolysin synthesis)	*Simon* et al.,1995
Extracted DNA from a variety of NCTC *Listeria* species	*hlyA* gene	*Bickley* et al., 1996
Listeria species isolated from various milk production farms	Enterobacterial repetitive intergenic consensus (ERIC) sequences and repetitive extragenic palidromic (REP) sequences	*Jeršek* et al., 1996

2.4.2.3 Ribosomal genes used for the detection and identification of *Listeria*

Classical bacterial phylogenetic classification was initially based on the analysis of metabolic properties (*Sallen* et al., 1996). However, molecular analysis of phylogenetic markers such as 16SrRNA and 23S rRNA resulted in much more accurate classification. At present 16SrRNA genes are the principle source of phylogenetic information and are also useful for the detection and identification of various pathogens (*Wang* et al., 1991, *Lin* and *Tsen*, 1995; *Lin* and *Tsen*, 1996). The advantages of using ribosomal genes (especially 16SrRNA) as molecular tools for identification are that they are present in every living cell, their function is highly conserved and they have both conserved and variable regions which is beneficial for studying relatedness within species and between genera (*Sallen* et al., 1996). In addition, a large variety of sequences of various genera and species are available in various databases, 16SrRNA genes are also smaller than 23S rRNA genes and do not have complex secondary structures. The main drawback of using 16SrRNA genes as a tool for identifying *Listeria* is that the sequences within species are so similar that one is not able to differentiate between them. However, *Herman* et al. (1995) partially overcame this obstacle by developing multiplex primers that were specific for *Listeria* (16SrRNA gene primer set) and

L.monocytogenes (*iap*-gene). Another way to identify *Listeria* is to amplify the internal spacer region of ribosomal genes. *Drebót* et al. (1996) and *Graham* et al. (1996) found that this was a useful region to amplify since the sizes of some of the amplicons varied and thus differentiation between species was possible. They also found that they could discriminate between *L.monocytogenes* serotype 4b and serotype 1/2b (epidemic outbreak strains) because of the presence of an additional 350bp amplicon. Both these primer sets are more specific in identifying *Listeria* than the classical methods.

2.4.3 Commercial kits for the identification of *Listeria*

Although conventional methods for the identification of *Listeria* have improved, these methods are still labourious and time-consuming. Various kits have been developed which identify *Listeria* on the basis of its ability to utilize specific carbon sources (BIOLOG, API, Micro-ID), elicit an immunological response (Microscreen *Listeria*, Listerscreen) and its genetic structure (Accuprobe).

2.4.3.1 Phenotypical identification of *Listeria*

2.4.3.1.1 BIOLOG GP test plates

This system consists of a 96-well microtitre plate with the redox dye (tetrazolium violet), suitable nutrients and the test carbon source. As the bacterium oxidizes a given carbon source, there will be increased respiration, which results in the reduction of the colourless tetrazolium dye to a purple formazan (*Bochner*, 1984). If the cells cannot oxidize the carbon source, the contents of the well will remain colourless. After incubation, a pattern develops with purple and clear wells, since this pattern is due to modification by respiration, this pattern is known as a metabolic "breathprint". Initially, the BIOLOG plates were developed for gram negative aerobes. However, recently microtitre plates have been developed for yeasts and gram positive bacteria. However, *Praphailong* et al. (1996) found limitations of the system to be its lack of reproducibility and exclusion of important foodborne yeasts from being detected. Only 68% (49/72) of the yeast strains were correctly identified using the BIOLOG YT plates. Since the database was mainly developed for gram negative bacteria, the variety of gram positive bacteria such as *Listeria* is limited. The "breathprints" of indigenous *Listeria* are different to type strains and

therefore consistent identification is difficult.

2.4.3.1.2 API *Listeria*

API *Listeria* is a 10-test strip that was developed for the biochemical identification of *Listeria* at the genus, species and subspecies level. *Billie* et al. (1992) found that 85% of the *Listeria* isolates were correctly identified even if they had some atypical characteristics. The advantage of this test is that the results are available within twenty-four hours and no haemolytic test is needed. In addition, *L.monocytogenes* (pathogenic) can be distinguished from the non-pathogenic strain *L.innocua.*

2.4.3.1.3 Micro-ID system

This system was designed to identify *Listeria* isolates to a species level within twenty-four hours. Each unit consists of 15 tests and the CAMP reaction. *Listeria* species are distinguished by the results on the CAMP test as well as a five digit octal code supplied by the manufacturer. This system identified 98.8% (409 out of 414 *Listeria* strains) compared to conventional biochemical tests (*Bannerman* et al., 1992). However, enterococci were incorrectly identified as *L.grayi*, *L.murrayi* or *L.innocua.*

Beumer et al. (1995) reported that in some cases the reading of the biochemical tests on the Micro-ID unit was difficult. This problem could be overcome by initially screening for catalase production, motility and gram positive rods. The other shortcoming of the system is that it must be done in conjunction with the CAMP test.

2.4.3.1.4 Microscreen Listeria

Microscreen *Listeria* is a latex agglutination test that presumptively identifies *Listeria* in enrichment broth and confirms the identification of *Listeria* on solid selective media. Latex particles are coated with polyvalent antisera prepared against purified flagellin proteins of *L.monocytogenes* (antigens A,B and C) and *L.grayi* (antigen A). If *Listeria* is present in the sample, agglutination occurs, resulting in white clumps. The advantages of this system are that it is quick, easy to perform and also reported to detect all the *Listeria* tested (*Beumer* et al., 1995).

2.4.3.1.5 Listerscreen Method

This is a method for the detection of *Listeria* using immunobeads supplied with the test kit Listerscreen. *Listeria* cells bind to the antibody -coated beads and are plated onto the selective media, PALCAM. After incubation, either classical biochemical tests or more rapid genotypical tests can be performed. Immunocapturing must be followed by culturing on selective agar as members of the genera *Corynebacterium* and *Erysipelothrix*, which are phylogenetically closely related to *Listeria* are also able to bind to the immunomagnetic beads. The advantage of this system is that it can be used for both food and environmental samples (*Avoyne* et al., 1997).

2.4.3.2 Genotypical kits for the detection and identification of *Listeria*

2.4.3.2.1 Accuprobe

The Accuprobe *L.monocytogenes* identification reagent kit is a non-radioactive, chemiluminescently labelled probe that hybridizes to a unique 16SrRNA region specific for *L.monocytogenes*. An overnight culture is lysed and the probe is added to this mixture. The bound probe is detected via a chemiluminescence reaction. This light emission is measured with a luminometer and converted into relative light units (RLU). A RLU reading of greater that 50 000U is considered to be positive. *Beumer* et al. (1995) found this method to be accurate, easy and rapid for the identification of *L.monocytogenes*.

2.5 Ecophysiology of *Listeria*

Listeria have a wide variety of habitats in which they can not only survive but also grow. However, this survival and growth is dependant on not only the habitat but also external parameters.

2.5.1 The optimum growth parameters for *Listeria*

The temperature range for growth is 1°C to 45°C with the optimum growth temperature between 30°C and 37°C. Usually strains of *Listeria* do not survive heating at 60°C for 30min. The pH

range is between pH 6.0 and pH 9.0. Growth occurs in broth supplemented with 10% NaCl. The water activity range is 0.932 and 0.942. They are aerobic or facultative anaerobes that are able to survive carbon dioxide concentrations between 50 to 80% (*Seeliger* and *Jones*, 1986).

2.5.2 Habitat of *Listeria*

2.5.2.1 Incidence of *Listeria* in the environment

2.5.2.1.1 Soil and water

Listeria is widespread in nature, occurring in soil, water and vegetation and thus frequently encountered by humans and animals on a daily basis (*Lovett* and *Twedt*, 1988; *Weis* and *Seeliger*, 1975). *Listeria* have been isolated from the intestinal tract of at least 35 mammals, 17 birds, ticks, trout, crustaceans, stream water, silage and soil (*Blenden* and *Szatalowicz*, 1967). *Welshimer* (1960) found *Listeria* could survive in fertile soil that was protected from evaporation for 295 days with only a slight decrease in the initial numbers. In a study in West Germany in 1975 (*Weis* and *Seeliger*, 1975) 194 *L. monocytogenes* strains were isolated from soil and plant samples, deer and stag faeces, mouldy fodder and wildlife feeding grounds and from the intestinal tract of most wild birds. Serovars 4b and 1/2b were predominant. They found that *Listeria* species were most commonly isolated from surface soil of uncultivated land (20 out of 39 samples) and the survival and growth in mud was also reported. Lowest numbers and counts were obtained from land or meadows used for cultivation of maize and corn. Nonetheless, it has also reported that higher counts can be obtained from cultivated land in which animal manure was used as fertilizer. Since *Listeria* occur in high levels not only in wildlife feeding grounds and the intestinal tract of wild birds, but also in uncultivated land and as part of the plant soil interaction, the question whether its natural habitat is soil or the intestinal tract of animals and humans remains unanswered. *Weis* and *Seeliger* (1975) summed it as follows :

"It seems unlikely that wild animals and birds are the only sources responsible for the distribution of *Listeria* in nature. The high incidence of *Listeria* in plant and soil samples would indicate that *L.monocytogenes* and nonvirulent varieties exist as saprophytes in soil and on plants."

2.5.2.1.2 Sewage and wastewater

The distribution of *Listeria* species in water is influenced by the type of water system, the levels of indigenous microflora and the level and type of pollution from animals and urbanization. *Bernagozzi* et al. (1994) found the predominant *Listeria* species in river water to be both *L.monocytogenes* and *L.innocua* (both 40%), while in treated and untreated sewage *L.monocytogenes* was the predominant species (62.5%). *Watkins* and *Sleath* (1980) also found *L.monocytogenes* in sewage usually in excess of *Salmonella* and on two occasions *L.monocytogenes* was found in the absence of *Salmonella.* There was a high correlation between faecal coliforms and *Listeria* in river water. *Colburn* et al. (1990) have shown that a specific serotype predominates in river water that is contaminated by sewage effluent or when domesticated animals grazed nearby. In contrast, *Frances* et. al. (1991) found that the predominant *Listeria* species in surface water of ponds and lakes was *L.seeligeri*. Both *Bernagozzi* et al. (1994) and *Frances* et al.(1991) agreed that *L.seeligeri* was an indigenous saprophyte in water.

Sludge provides a beneficial environment for the growth of *L.monocytogenes* and this organism has been found in concentrations greater than 10^4 cells/ml. Thus, using sewage sludge or waters receiving sewage effluents as irrigation water for ready-to-eat products such as lettuce and tomatoes is a hazardous practice. Growth of *Listeria* during wastewater treatment is favoured by biological oxidation (*Lovett* and *Twedt*, 1988).

2.5.2.2 The incidence of *Listeria* in raw and processed food products

2.5.2.2.1 Dairy products

2.5.2.2.1.1 Raw milk

The incidence of *Listeria* in bulk raw milk tanks as well as the raw milk receiving rooms is low (*Fenlon* and *Wilson*, 1988; *Charlton* et al., 1989). *Fenlon* et al. (1994) found between 1 and 35 cells/ml. Recovery of *L.monocytogenes* from raw milk was also found to be seasonal, the distribution was sporadic and not related to hygiene or the feeding of silage (*Slade* et al., 1989; *Fenlon* and *Wilson*, 1988; *Charlton* et al., 1989).

In the dairy processing plants tested, *Listeria* species were isolated more frequently from fluid milk product plants than from frozen milk product plants. *Listeria* were isolated from fluid milk products (12.9% of the time and 30.2% of the isolates were *L.monocytogenes*)(*Charlton* et al., 1989). Various authors (*Fenlon* and *Wilson*, 1988; *Charlton* et al., 1989) found it unusual that the predominant area for isolating *Listeria* was in fluid milk systems that are usually closed. Usually frozen milk products and cheeses have a greater exposure to environmental contamination. Thus, one would expect higher levels of *Listeria* contamination in these systems than in the fluid milk systems.

Listeria mastitis in cows is rare, but it poses a health threat to industries that use raw milk. For example, traditional and industrial fermented milk are part of the diet of both Zimbabwean babies and the elderly. It is thus imperative that the raw milk is not contaminated with *L.monocytogenes*. The risk is due to both persistence of infection and numbers of contaminating organisms (*Dalu* and *Feresu*, 1996). It has been shown that mastic cows can shed $2x10^3$ - 2×10^4 cells/ml and excretion still occurs 3 months after clinical symptoms have disappeared (*Bourry* et al., 1995). To prevent contamination of bulk milk, early detection of *Listeria* mastitis in cattle herds is essential. Naturally infected hindquarters were contaminated with between 1.5 and 9.2 *L. monocytogenes* cells per phagocyte (*Dalu* and *Feresu*, 1996). These high figures could explain persistence of infection and scant rate of recovery. Additionally, the antibiotics used for intramammary treatments are less effective against intracellular *L.monocytogenes (Fenlon* et al., 1994).

2.5.2.2.1.2 Cheese

Traditionally, the sensory characteristics of some soft cheeses are enhanced by the use of unpasteurised milk which allows *L.monocytogenes* to survive and multiply during ripening. An example of a product manufactured with unpasteurized milk, is Italian Mozzarella cheese which is not only produced from raw milk but is also enjoyed as soon as possible after the manufacture of the cheese (*Villani* et al., 1995). Recently some Camembert cheese has been produced by replacing the classical mesophyllic starter strains with lactic acid produced species such as eg. *Lactococcus thermophilus*. This modification results in the pH of the curd before salting not going below pH 5, which is within the pH growth range of

L.monocytogenes (*Wan* et al., 1996). *Papageorgiou* and *Marth* (1988a) reported that *L.monocytogenes* was able to grow during feta cheese making as well as survive during ripening and storage. Both strains of *L.monocytogenes* used in this experiment could survive in feta cheese for more than 90 days even at a low pH of 4.3 and at a temperature of 4°C. This demonstrated that cheese held at or above 1-7°C for 60 days cannot be assured of being free of viable pathogens.

Acidification (pH 4.6 - 4.9 during first 20 days) plays a critical role in reducing the number of *L.monocytogenes* in blue cheese. It is suspected that high proteolysis and lipolysis activity by *P. roqueforti* results in free fatty acids in blue cheese which inactivates *L.monocytogenes* during the first 50 days of ripening. After 50 days, an increase in pH allowed the one strain, Scott A to survive. An isolate from Mexican-style cheese outbreak, *L.monocytogenes CA*, is less tolerant of low pH conditions and becomes gradually inactivated under these conditions. Strangely, growth of *L.monocytogenes* was not observed during ripening even though the pH was within the growth range of *L.monocytogenes* (*Papageorgiou* and *Marth*, 1988b). The authors believe that this was due to the high salt content (4.26 - 4.28) but additionally also the presence of free fatty acids, methyl ketones and corresponding ketones derived from the ß- oxidation pathway, inhibited growth in feta (*Papageorgiou* and *Marth*, 1988a). *Papegeorgiou* and *Marth* (1988b) showed that *L.monocytogenes* could grow in blue cheese during the initial ripening stage until pH 5.0.

2.5.2.2.2 Protein-rich animal foods

2.5.2.2.2.1 Incidence in seafoods

Although seafood has been epidemiologically linked to an outbreak in New Zealand, no conclusive evidence has been found (*Farber* and *Peterkin*, 1991; *McCarthy* et al., 1989). *Fuch* and *Surendran* (1989) examined 35 tropical fish and fish products for the presence of *L.monocytogenes*. This organism was found in 3 out of the 10 fresh samples and in 5 of 14 frozen samples. No *Listeria* were detected or isolated from dried and salted fish. The authors suggested that the high salt content (15-20%) and the U.V. irradiation when fish was sundried for 2 days resulted in vegetative cells being destroyed. However, the sundried fish was at risk of being faecally contaminated with *Listeria* by wild birds (*Fenlon*, 1985).

McCarthy et al. (1989) reported recovery of *Listeria* from raw shrimp but not from shrimp boiled for 1-5min. These authors believed that the inability to recover *Listeria* was due to the heat and freeze-thaw combination which placed lethal stress on *Listeria* in naturally contaminated shrimp. In contrast, *Farber* et al. (1991) recovered *L.monocytogenes* from unfrozen cooked shrimp, from raw, spiced shrimp that was held 20°C for 90 days, but not from frozen cooked shrimp. Besides shrimp, *L. monocytogenes* was also shown to multiply in cold salmon during storage at 4°C and 10°C. The ability of *L. monocytogenes* to grow at storage temperatures reinforced the hypothesis that initial contamination of the raw product should be avoided (*Guyer* and *Jemmi*, 1991).

2.5.2.2.2.2 Chicken and chicken products

Farber et al. (1989) reported that the range of contamination of chicken is between 12-60%. *Dykes* et. al. observed the presence of *Listeria* on the neck skins of poultry in a South African poultry plant after evisceration. They found that the rubber fingered pluckers were contaminated with *Listeria* species but not the neck skins before evisceration. They deduced that contamination occurs during the scalding process, as the scalding tanks are heavily contaminated with faeces. The microflora of the chicken faeces includes *Listeria* species which are then transferred to the chicken feathers and finally contaminate the rubber fingered pluckers. However, in contrast to these findings, other authors could not isolate *Listeria* from the scalding tanks *(Ojeniyi* et al., 1996). In addition, they reported that chickens were not a natural reservoir for *L.monocytogenes.* However, intestinal carriage could be due to environmentally contaminated food or water. They concluded that contamination was thus due to the ineffectiveness of disinfection of the processing line. Eggs have also been shown to harbour *Listeria* species. Egg-shells are normally contaminated with *Listeria* species via soil and faeces. However, no *Listeria* have been isolated from pasteurised eggs and egg products (*Moore* and *Madden*, 1993).

2.5.2.2.2.3 Meat and meat products

According to *Farber* and *Peterkin* (1991) meat is usually surface-contaminated by *L.monocytogenes.* Worldwide the most prominent serovar found in meats has been serotype 1 although serotype 4b was

isolated in Britain in 1989 from contaminated paté (*McLaughlin*, 1991). Since most listeriosis outbreaks have involved serotype 4b, some researchers believe meat cannot be involved in listeriosis outbreaks (*Johnson* et al., 1990). Although no listeriosis epidemics has been linked to meats (*Glass* and *Doyle*, 1989; *Shelef*, 1989), many sporadic cases indicate the possibility that meat can be a transmission agent for *L.monocytogenes*. *Johnson* et al. (1990) reported that human non pathogenic *Listeria*, especially *L.innocua*, are more predominant in meat and meat products than *L.monocytogenes*. According to *Bunčić* (1991) approximately 69% and 80% of all Yugoslavian mince meat contained *L.monocytogenes* and *L.innocua* respectively, although neither of these species were detected in the deeper parts of carcass meat. *Farber* et al. (1989) also observed that more than 80% of all isolates from ground beef belonged to serotype 1. In a study done in New Zealand, 30-65% of work surfaces and knives tested were contaminated by *L.monocytogenes* while 15-40% were contaminated by other *Listeria* species (*Johnson*, 1990). Johnson *et al.* (1988) reported survival but not growth of *L.monocytogenes* in ground beef held at 4 °C for 2 weeks. They deduced that contamination of ground beef by *Listeria* could be due to both contamination of cutting equipment and utensils and the inclusion of meat trimmings such as the lymph nodes, although this was not proven.

Although there have only been epidemiological links between sporadic outbreaks of listeriosis involving uncooked hot-dogs, processed meat such as sausage cannot be discounted as a vehicle for foodborne listeriosis (*Farber* et al., 1989; *Johnson* et al., 1990). Even though processed meats, eg. sausages, have not been linked directly to listeriosis, major economic losses have been experienced by various companies through the recalling of products for safety reasons. *Farber* et al. (1993) demonstrated the growth of *L.monocytogenes* in German, American and Italian-style sausages. Both German and American-style sausages were prepared with the starter cultures *Pediococcus pentosaceus* and *Pediococcus acidilactici*. A reduction of 1-2 logs and 5 logs of *L.monocytogenes* respectively was observed. The greater reduction in *L.monocytogenes* numbers in American compared to German-styled sausage could be due to *L.monocytogenes* being more sensitive to PA-1 (bateriocin produced by *P.acidilactici*) than *P.pentosaceus* FBB63 (*Pucci* et al., 1988). This bacteriocin was shown to be effective in high protein and fat

environments. *Farber* et. al. (1993) also found that *L.monocytogenes* numbers in Italian-style sausage that had no starter culture, increased during fermentation, remained constant during drying and decreased slightly during a 4 week storage period at 4°C. *Berry* et al. (1990) however, observed no growth and a slight decrease in numbers in summer sausage made without a starter culture. These differences could be due to the variations in chemical and physical properties of various sausages (*Farber et al.*, 1989). *Listeria* spp. are also affected by temperature of storage, pH, sodium chloride concentration, type of tissue, background microflora and method of preservation used (*Johnson* et al., 1990). *Messina* et al. (1988), reported that liquid smoke preparations that are used in products such as viennas have an antimicrobial effect against *L.monocytogenes*. This anti-listerial activity is possibly due to the effect of phenols on *Listeria*.

2.5.2.2.3 Vegetables

Beuchat and *Brackett* (1990a) reported *L.monocytogenes* to be widely distributed on plant vegetation due to contamination by animal manure, decaying vegetation, soil, water and effluents from water treatment facilities. Vegetables have been involved in one listeriosis outbreak and epidemiologically linked to another. In an outbreak of listeriosis in Boston in 1979, salad vegetables were epidemiologically linked to the outbreak. *Beuchet* et al. (1990), confirmed that salad vegetables such as lettuce, broccoli, cauliflower and asparagus are low acid foods and thus are able to support the growth of *L.monocytogenes* at refrigeration temperatures. *Heisick* et al. (1989) also isolated *L.monocytogenes* serotype 1 from fresh market produce including raw cabbage, cucumbers, potatoes and radishes. However, high acid content vegetables, such as tomatoes are antagonistic to the growth of *L.monocytogenes* (*Beuchat* et al.,1990) . The predominant acid in tomatoes is acetic acid which has been shown to be particularly inhibiting to the growth of *L.monocytogenes*. Carrots (*Beuchat* and *Brackett*, 1990b) have also been shown to exhibit an inhibitory effect. This antilisterial effect is lost when the carrots are cooked. Although the authors are not sure what the inhibitory component is, they have suggested that certain phenolic compounds in carrots are toxic to *Listeria*. In a listeriosis outbreak in the Maritime Provinces in Canada in 1981, cabbage contaminated with manure from sheep that ovine listeriosis, was stored at refrigeration temperatures. This

storage resulted in the multiplication of *Listeria* and contamination of the final product, coleslaw (*Farber* and *Peterkin*, 1991).

2.5.3 Control measures against *Listeria*

The trend towards a 'hurdle' approach for food preservation and safety is becoming increasingly popular. The basis of this preservation and safety method is to create as many hurdles or barriers which pathogens and spoilage bacteria have to overcome if they are to survive and grow in a particular food product. These barriers include both intrinsic (eg. heat temperature, pH, salt concentration, preservatives) and extrinsic (eg. storage temperature, packaging atmosphere, competing bacteria) factors (*Muriana*, 1988).

2.5.3.1 Intrinsic parameters

2.5.3.1.1 Temperature as a control method against *Listeria*

Temperature is an important factor in the control of *L. moncytogenes*. However, controversy surrounds both the thermotolerence and psychrotolerence of *L. monocytogenes*. Studies of other psychotrophic bacteria have suggested that changes in the fatty acid composition of membrane polar lipids may enable maintenance of membrane integrity over a range of temperatures (*Jones* et al.,1997). Temperature-induced change in bacterial fatty acid have been described. These include an increase in unsaturation, a decrease in chain length, a reduction in the amount of cyclic acids and procurement of lipids from complex growth media. In addition, they have reported the presence of a unique glycolipid related to psychotolerence. The study supported the hypothesis that the psychotolerence nature of *L. monocytogenes* is due to its ability to modify lipid composition. *Bayles* et al. (1996) have reported the presence of 12 cold shock proteins (Csp) induced in *L. monocytogenes* when the temperature was lowered from 37 °C to 5 °C. Additionally, cold acclimation proteins (Cap) were produced when *L. moncytogenes* 104035 was held at 5 °C. Phan-thanh and Gorman (1995) found 32 Csp in another *L. monocytogenes* strain, the authors believe that finding only 12 Csp could be a strain variation or a conservative estimate. These proteins had a dual nature being both Cap and Csp depending on the temperature of the growth environment.

As far as thermotolerence is concerned, the effectiveness of pasteurization remains controversial since an outbreak in Massachusetts of listeriosis linked to the consumption of pasteurized milk. *Farber* and *Peterkin* (1991) suggested that since the proper pasteurization procedure was followed, the intracellular location of *L. monocytogenes* in the bovine lymphocytes protected the organism. *Lou* and *Yousuf* (1997) found that environmental stresses influenced the resistance of *Listeria* to processing temperatures.

2.5.3.1.2 pH as a control measure

Listeria grew optimally at neutral or alkaline pH. *Patchett* et al. (1996) found cells grown at 30°C were more resistant to acid stress at pH 2.5 than cells grown at 10°C. Thus, the tolerated pH is dependant on temperature, oxygen tension, salt concentration. Various authors have shown that the type of acid affects the degree of inhibition of *L.monocytogenes*. Hypochloric acid is less inhibitory to *L.monocytogenes* than organic acids such as acetic, citric or lactic acids (*Farber* and *Peterkin*, 1991).

2.5.3.1.3 Salt concentration as a control method

L.monocytogenes is relatively resistant to increased salt concentration within a defined pH range. *L.monocytogenes* has been shown to grow in 10% salt at 35°C and at neutral pH (*Jones*, 1990).

2.5.3.2 Antimicrobial substances which control the growth of *Listeria*

2.5.3.2.1 Antibiotics, lantibiotics and bacteriocins

The group of peptides active against gram positive bacteria can be divided into three groups, namely, antibiotics, bacteriocins and lantibiotics. In the natural habitat, *Listeria* are exposed to various antibacterial agents produced by competing microflora. Generally ampicillin, erythromycin, getamicin, trimethophrin or rifamycin alone or together in different combinations are effective against *L.monocytogenes* (*Hof* et al., 1997). Usually ampicillin or benzyl penicillin are the antibiotics of choice. However, patients allergic to penicillin are treated with tetracyline and erythromycin. In a patient with Meningoencephalitis, a strain of *L.monocytogenes* exhibited resistance to chloramphenicol, erythromycin, streptomycin and tetracycline. The resistance genes are carried on 37kb plasmid (pl P811). This plasmid is

self transferable to other *L.monocytogenes* cell, enterococci, streptococci and *Staphylococcus aureus*. Extensive sequence homology between p1P811 and pAMB1, which is found in streptococcic and suggests that enterococci multiple antibiotic resistance in *L.monocytogenes* is as a result of conjugative transfer. This plasmid from gram positive cocci, enterococci and streptococci the commonly found in the intestinal tract of man and animals . Transfer of multiple resistance from *L.monocytogenes* to other strains is a possibility but has not been extensively investigated (*Rota* et al., 1996).

Lantibiotics and bacteriocins are distinguished by an intramolecular ring moiety that contains a p amino acid lanthrionne eg. nisin. *Davies* and *Adams* (1994) and *Davies* et al.(1996) reported that nisin is once again attracting interest as a natural biopreservative to control vegetative organisms, especially the gram positive pathogen, *L.monocytogenes*. The target of nisin is the cytoplasmic membrane. Nisin becomes incorporated into the cytoplasmic membrane, produce pores which result in cell lysis and death. Bacteriocins are GRAS (Generally Regarded As Safe) substances produced as by- products during growth of starter culture bacteria. In addition to their usefulness in inhibiting spoiling bacteria and pathogens (such as *L.monocytogenes*) bacteriocins also extend the shelf-life and enhance the flavour of the food products. The use of bacteriocins have resulted in 1 to 3 log reduction in *Listeria* populations. Although this is not acceptable as a primary preservation method, in context of the 'hurdle concept', bacteriocins create an additional 'barrier' for *L. monocytogenes* to overcome. *Muriana* (1988) extensively reviewed lactic acid bacteria that have an antilisteritic effect. *Pediococcus acidilactici* PAO1 is also used as a biopreservative which as an inhibitory action against *L.monocytogenes* over a wide pH and temperature range. Since *Pediococcus acidilactici* PAO 1.0 is a traditionally used starter , it has GRAS status. Additional *P. acidilactici* PAO 1.0 was shown to have a synergistic effect with lactic acid when in a food system (*Pucci* et al., 1988). They may safeguard food products such as Camembert cheese from high levels of *L.monocytogenes* contamination.

Amongst the non-lactic acid bacteria, *Enterococcus spp.* are the most widely recognised and characterised. Enterococci produce a variety of bacteriocins (enterococcins) which are active against foodborne pathogens

such *as L.monocytogenes*. Various characteristics of enterococcins make them ideal for the application as biopreservation in food include its heat stability, stability over wide pH range, broad spectrum activity, generally harmless and does not affect the growth of starter culture. However, enterococcins produced by *Enterococcus faecium* and *Enterococcus faecalis*, do not have as wide a spectrum as nisin (*Giraffa*, 1995).

2.5.3.2.2 Lactoperoxidase system

Denis and *Ramet* (1989) investigated the effect of the lactoperoxidase system in UHT milk, soft cheese and typticase soy broth. Although the lactoperoxidase system was shown to be effective against *L.monocytogenes*, a limitation was that the system did not take other factors such as food components, water activity and microbial load, into account.

2.5.3.2.3 Control of *Listeria* by plant metabolites

Spices are seldom used for their antimicrobial properties, since the effective doses needed are usually not organoleptically sound. *Pandit* and *Shelef* (1994) screened 18 spices in culture media to determine their antilisterial effect at a concentration of 1%. Of the spices tested, only rosemary and cloves were antilisterial.

2.5.3.2.4 Synthetic substances for controlling *Listeria*

Listeria have been shown to be susceptible to commonly used food preservatives which include nitrate, sorbic and benzoic acid. However, these preservatives do not function in isolation, but their action against *Listeria* is enhanced by other parameters such as temperature and pH. The parameters for the optimal functioning of both sorbic and benzoic acid are at temperatures of 4°C and pH of 5.0. Nitrite on its own is not inhibitory to *L.monocytogenes* but in conjunction with other intrinsic factors such as temperature, pH and salt concentration, *L.monocytogenes* numbers are reduced (*Doyle*, 1988).

2.5.3.2.5 Control through microbial competition

Microbial competition in transmission vectors as well as the hosts of *Listeria* plays a pivotal role in the

infection cycle. *Czuprynski* (1994) found that the gastrointestinal flora in mice affected the virulence of *L.monocytogenes* since it was outcompeted by other gut flora. In food products, the presence of starter cultures, *Pseudomonas*, *Yersinia* and *Escherichia coli* all influence the growth of *Listeria*. *Listeria* were shown to decrease in the presence of *Lactobacillus plantarum* while increasing when grown together with *Pseudomonas fluorescens* (*Johnson*, 1990). *Pucci* et al. (1988) observed that acid produced by starter cultures as well as bacteriocins suppresses the growth of *L.monocytogenes* in dairy products.

Marshall and *Schmidt* (1987) evaluated the growth of *L.monocytogenes* in whole, skim and non-dairy milk in the presence of co-inoculated pseudomonades of dairy origin. They found that the growth of *L.monocytogenes* was increased in milk inoculated with both pseudomonads and *L.monocytogenes*. Furthermore, the doubling time of *L. monocytogenes* at 10°C was decreased by up to three hours when *Pseudomonas* species were present. It is hypothesized that the proteolytic activity of pseudomonads has a stimulatory effect on *Listeria monocytogenes* and other organisms. *Guyer* and *Jemmi* (1991) found that both pseudomonads and *E.coli* had stimulated the growth of *Listeria* in fish. In contrast, *Budu-Amoako* et al. (1992), found that *Yersinia enterocolitis* in whole milk had an inhibitory effect at 10 and 22°C but not at 4°C. Since a pH of 6.16 should not limit the growth of *L.monocytogenes* , thus a possible reason for the inhibition could be that *Y.enterocoitica* is outcompeting *L.monocytogenes*. However, no inhibition was observed in skim milk, irrespective of the temperature used. *Guyer* and *Jemmi* (1991) also showed that the bacterial load of raw products was significant in inhibiting the growth of *Listeria*. The relationship between the number of *L.monocytogenes* and aerobic plate counts remain controversial. *Leistner* (1978) noted that if the total plate count was below 10^5 colony forming units (CFU) the incidence of *Listeria* were minimal. In contrast, if the total aerobic plate counts are higher than 10^7 CFU, no *Listeria* were found. This phenomenon can possibly be due to increased competition by other microbes, the ratio of *Listeria* to other competitors and the recovery method used.

2.5.3.3 Measures by the bacteria to resist control

2.5.3.3.1 Protection offered by biofilm formation

Listeria are able to attach to various surfaces including stainless steel, glass, teflon, nylon and rubber (*Lee*

and *Frank*, 1991). Attachment to these surfaces can take place after short periods of time as well as at refrigeration and ambient temperatures (*Ralóvich*, 1992; *Blackman* et al., 1996). *Listeria* are also able to grow and survive in multispecies biofilms. *Blackman* et al. (1996) found that attached cells were more resistant to sanitizers (chlorine, iodine, acid anionic and quaternary ammonium) and higher concentrations of sanitizers were needed in areas where *Listeria* was part of a biofilm. *Krysinski* et al.(1992) found that resistance to the biocide also depended on the surface of attachment. *Blackman* et al. (1996) hypothesised that the creation of *Listeria* biofilms could result in contamination of an entire food-processing plant due to the production of aerosols during the cleaning of these surfaces.

2.5.3.3.2. Resistance to antibiotics

Listeria was initially thought to be sensitive to all antibiotics against gram positive bacteria. However, over 70 antibiotic resistant strains have been isolated during both sporadic and epidemic outbreaks of listeriosis. *Listeria*- acquired antibiotic resistance is thought to be due to two types of movable genetic elements, namely, self-transferable plasmids and conjugative tranposons (*Charpentier* et al., 1995). Screening for *Listeria* in raw milk samples, *Slade* and *Collins-Thompson* (1990) found that only 2 out of 26 *L.innocua* isolates were resistant to tetracycline while all *L.monocytogenes* isolates as well as one *L.welshemeri* isolate were sensitive to all nine commonly prescribed antibiotics. They also found that in this case, tetracycline resistance was chromosomally and not plasmid mediated. *Facinelli* et al. (1991) also isolated tetracycline resistant *L.innocua*. They hypothesised that tetracycline resistance was aquired in the gut of animals and humans (where *L.innocua* is commonly found) as a result of increased use of tetracycline in animal (especially chickens) husbandry. They also found multiple resistant *L.monocytogenes* strains from mozarella cheese. Resistance in this case was found to be plasmid mediated. In a study using 1100 isolates, *Charpentier* et al. (1995) found 37 tetracycline and minocycline resistant *L.monocytogenes* strains. All other strains were sensitive to commonly used antibiotics such as ampicillin and gentamycin. Thus they concluded that although the incidence of resistance remains relatively low, the fact that there are resistant *L.monocytogenes* strains, should be taken into account before treatment is prescribed.

2.5.3.3.3 Resisting control through adaptation to environmental stresses

Foodborne pathogens are constantly stressed in the food processing environment by thermal treatments, exposure to hydrogen peroxide, exposure to acid produced by starter cultures and high concentrations of sodium chloride. This 'hurdle" approach is commonly used in the food processing environments to inactivate pathogens such as *Listeria*. However, *Lou* and *Yousef* (1997) have found that *Listera* that initially survive environmental stress are more resistant to lethal stresses in the food processing environment. This phenomenon is known as 'stress hardening'. Adaptation by *Listeria* to sublethal environmental stresses not only protected it from the same stress but also from other lethal stresses. Stress hardening could also explain the fact that there is a greater implementation of the hurdle approach in food industries but there is also an increase in foodborne illness. Thus, this sequential implementation of hurdles in the food processing environment must be re-evaluated to ensure food safety.

2.6 Conclusions drawn from the literature

Since the description of *Listeria* almost seventy-two years ago, various questions concerning the habitat, physiology and the target population have been answered. However, there are many questions regarding the ability to survive unfavourable conditions, pathogenicity and isolation methods.

A variety of not clearly understood characteristics allows this nonsporeformer not only to survive but also grow under/in adverse conditions such as extremes in temperature and salinity. Some *Listeria* are even adapted to survive unfavourable conditions in the human or animal host's cells. As an opportunistic pathogen, the target population, is not normally healthy individuals but the immunocompromised. This group includes neonates, the elderly, alcoholics, HIV-infected people and those on immunosuppressive drugs. Although a variety of pathogenetic genes (eg. *iap*, *prf*, *msp* genes) have been identified, the function of these genes and their precise role in the pathogenicity process is not clearly understood. A variety of isolation, detection and identification methods incorporating phenotypical or genotypical characteristics have been developed to isolate *Listeria*. Although there is currently a "zero-tolerance" for all *Listeria* in processed food products, most methods concentrate on the predominant human pathogen, *L.monocytogenes* thus excluding all other species. Therefore, the prevalence of *Listeria* in both nature and food-processing environment could be underestimated. Besides concentrating only on *L.monocytogenes*, many methods are limited to a certain type of system. Therefore a reliable all- inclusive method(s) are needed to determine all *Listeria* irrespective of the source.

Chapter 3

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Prevalence of *Listeria* in South African Blue-cheese as determined by conventional tests and PCR

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Listeria is a gram positive, non-sporing coccobacilli that occurs in various foods, the environment and in the intestinal tracts of various animals, birds and humans. Outbreaks of listeriosis due to the consumption of foods contaminated with *L.monocytogenes*, coupled with the high mortality rate and the risk posed to immunocompromised individuals have resulted in more focus being placed on *Listeria*-free products. Besides milk, soft cheeses have also been implicated in listeriosis outbreaks. In this study, locally produced blue cheese was tested for the prevalence of *Listeria*. Samples of Blue cheese were grown in UVM1 and then isolated using Listeria selective of Agar (LSA). Black colonies on LSA were presumed to be *Listeria* and further tested both genotypically (PCR) and phenotypically (BIOLOG). The primer set UnilisA and Lis 1B which detects the presence of the *iap* gene coding for the invasive associated protein was used to detect for *Listeria*. *Listeria* were confirmed phenotypically using the Biolog GP system. Presumptive *Listeria* isolates were obtained from all 24 cheese samples, 11 (45.8%) of which were both phenotypically and genotypically positive. The remaining 13 samples were all PCR negative and only 4 produced a Biolog pattern that matched sufficiently with the database to be confirmed as non- *Listeria*. Only one isolate (G21) was phenotypically related to the main cheese cluster but did not possess the *iap* gene. These results indicate that blue cheese regularly harbours *Listeria*. Thus, effective pasteurisation and the use of more hygienic techniques to eliminate the introduction of *Listeria* in the cheese-making process are necessary.

3.1 INTRODUCTION

Listeria is a gram positive, non-sporing coccobacilli that occurs in various foods, the environment and in the intestinal tracts of various animals, birds and humans (*Blendon* and *Szatalowicz*, 1967; *Fenlon*, 1985; *Welshimer*, 1960; *Weis* and *Seeliger*,1975). Although there are currently seven species belonging to this genus, attention has mainly been focussed on *Listeria monocytogenes* since it is the most common a human pathogen that causes listeriosis. Outbreaks of listeriosis due to the consumption of foods contaminated with *L.monocytogenes*, coupled with the high mortality rate and the risk posed to immunocompromised individuals have resulted in more focus being placed on *Listeria*-free products (*Farber* and *Peterkin*, 1991; *Gellin* and *Broome*, 1989; *Macgowan* et al., 1991; *Schlech* et al., 1983; *Jurado* et al., 1993). Various authors (*Fenlon*, 1985; *Fenlon* et al., 1994) have shown that there is a low incidence of *L.monocytogenes* in bulk milk tanks. In order to further decrease the risk of foodborne illness associated with raw milk, dairy industries use pasteurized milk. However, in 1983, in Massachusettes, a mortality rate of 29% was noted among adults and infants who had consumed a particular brand of pasteurised milk (*Farber* and *Peterkin*, 1991; *Schlech* et al., 1983). It was suggested that the bovine lymphocytes found in the milk protected the intracellular pathogen, *L.monocytogenes* from the pasteurization process (*Bourry* et al., 1995). In contrast, *Farber* et al.(1988) found that *L.monocytogenes* could not survive pasteurization.

Besides milk, soft cheeses have also been implicated in listeriosis outbreaks . The potential of *L.monocytogenes* to grow and survive in other soft cheeses such as feta, Camembert and blue cheese has also been investigated (*Papageorgiou* and *Marth*, 1988a; *Papageorgiou* and *Marth*, 1988b).

Since many questions regarding the prevalence and pathogenicity of the organism remain unanswered, this study was undertaken to determine the prevalence of *Listeria* in South African produced Blue cheese by conventional culture methods as well as by PCR using the primers (UNILISA and LIS1B) derived from the sequence for the *iap* (invasion associated protein) gene (*Bubert* et al., 1992).

3.2 METHOD AND MATERIALS

3.2.1 Sample collection

Twenty-four samples (*ca.*200g) of Blue cheese from a local producer were randomly sampled at various stages of ripening. Samples were collected in sterile Whirlpak bags, transported to the laboratory on ice and processed within 48 hrs.

3.2.2 Isolation of *Listeria* species

Twenty-five grams of Blue cheese was pummelled in 225ml of Listeria Enrichment Broth (CM856 + SR142, Oxoid, Basingstoke, U.K) in a stomacher for 2 min at medium speed. After 4 and 24 hrs incubation at 30°C, 1ml of the primary enrichment was serially diluted in sterile tap water, spread-plated onto duplicate plates of Listeria Selective Agar (LSA)(CM854 + SR140, Oxoid) and incubated at 30°C for 48hrs. After 24hrs, 1ml of the primary enrichment was also transferred to 4.5ml of 0.5% KOH, vortexed for 1min and spread-plated onto duplicate LSA plates. Colonies that were surrounded by black zones were purified on LSA for further characterization.

3.2.3 Reference strains used

The following type strains from the American Type Culture Collection were utilized as reference strains in all experiments: *L.monocytogenes* ATCC 15313, *L.monocytogenes* NCTC 7973, *L.ivanovii* ATCC 19119, *L. innocua* ATCC 33090, *L.seeligeri* ATCC 35967, *L. welshimeri* ATCC 35897, *L. grayi* ATCC 19120 and *Jonesia denitrificans* ATCC 14870.

3.2.4 Morphological tests

The morphology of one presumptive *Listeria* isolate from each plate was tested by using the gram stain and confirmed with the KOH method *(Powers*, 1995). All gram positive nonsporing rods were then streaked onto Tryptone Soya Agar(TSA)(CM356, Oxoid) and incubated overnight at 30°C.

3.2.5 Characterisation of isolates on Biolog GP test panels

All isolates were streaked onto BUGUM (Biolog Inc.,USA) supplemented with 0.5 % (v/v)of defribrinilated blood and incubated at 30°C for 24 hrs. A suspension of the overnight culture was made in 20ml of 0.8% NaCl and inoculated onto the Biolog GP identification test panels according to the manufacturers instructions. After 4 and 24 hrs incubation at 30°C, the plates were read using a multiscan microplate spectrophotometer at a wavelength of 590nm. The data was analysed using the Microlog software system (Biolog). Two-dimensional data plots were created and clustered in relation to its closest relatives.

3.2.6 Detection of the *iap* gene by PCR

The PCR was performed according to *Bubert* et al.(1992) with the following modifications: 0.5U Taq polymerase, 150μM dNTP mix and 1μl of crude bacterial lysate was added to a final volume of 50μl. The DNA amplification conditions were followed according to the protocol, but the annealing temperature was adjusted to 66°C. The PCR tubes were overlaid with 20μl of mineral oil and the PCR was performed using a Hybaid Omn-E thermal cycler. The resulting PCR products were seperated by horizontal electrophoresis in a 0.8% agarose gel and visualized by ethidium bromide staining and U.V. transillumination at 254nm.

3.3 RESULTS

Table 6. Phenotypical and genotypical results of the cheese isolates and the type strains identified by BIOLOG. Nine further isolates were negative for all tests

Strain no.	Source	BIOLOG GP	PCR of *iap* gene
A1	*J. denitrificans* ATCC 14870	-	-
B2	*L. ivanovii* ATCC 19119	+	+
B3	*L. monocytogenes* NCTC 7973	+	+
C4	*L. monocytogenes* ATCC 15313	+	+
C5	*L. monocytogenes* ATCC 15313	+	+
D6	*L. grayi* ATCC 19120	+	+
D7	Cheese 21	+	+
E8	*L. seeligeri* ATCC 35967	+	+
F9	Cheese 19	+	+
F10	Cheese 23	+	+
F11	*L. innocua* ATCC 33090	+	+
F12	Cheese 24	+	+
F13	Cheese 25	+	+
F14	Cheese 1	+	+
F15	*L. welshimeri* ATCC 35897	+	+
F16	Cheese 15	+	+
F17	Cheese 14	+	+
F18	Cheese 8	+	+
F19	Cheese 10	+	+
F20	Cheese 9	+	+
G21	Cheese 11	-	-
H22	Cheese 12	-	-
I23	Cheese 22	-	-
J24	Cheese 13	-	-

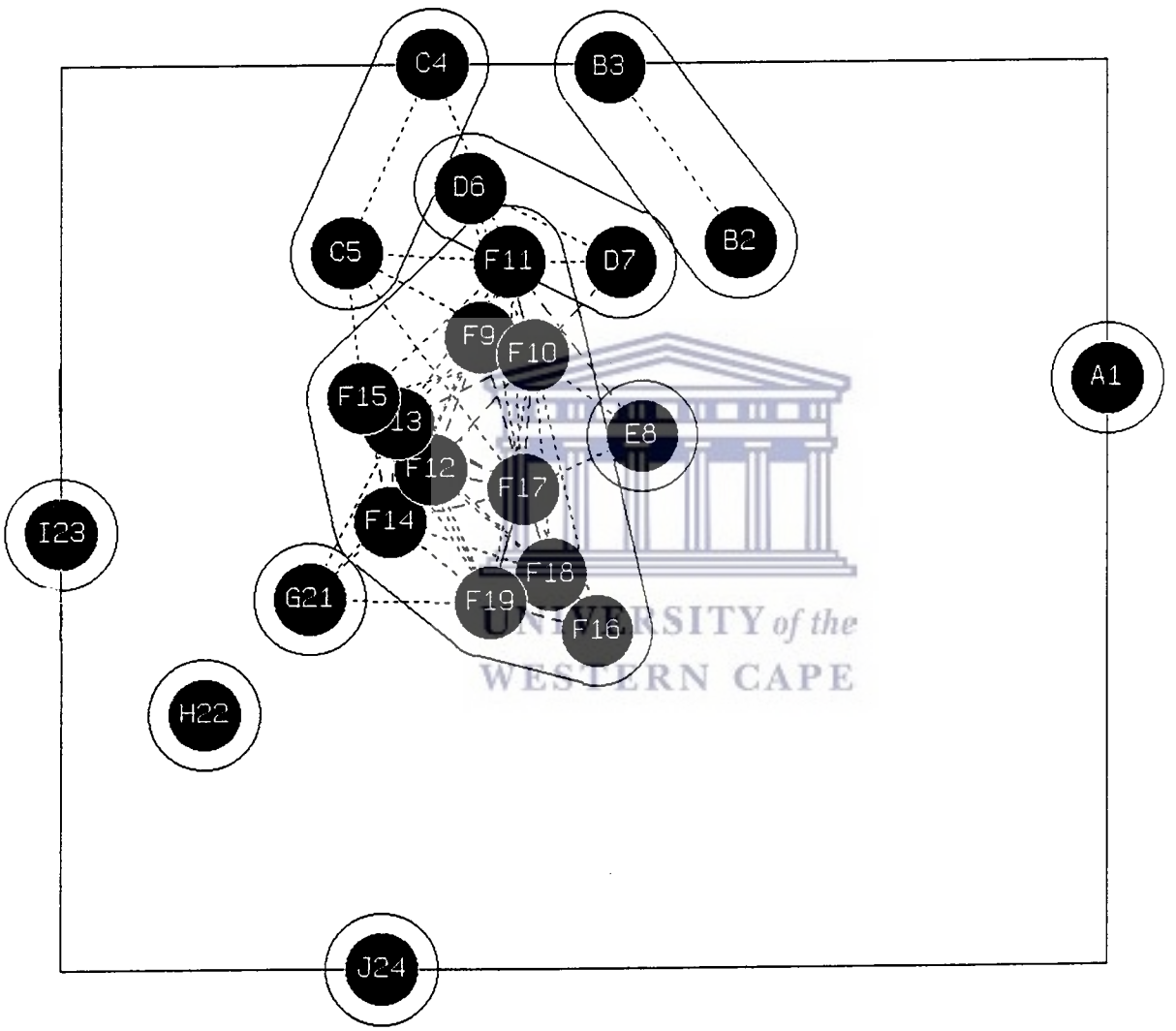

Fig. 3 Two dimensional representation of the relatedness of the blue cheese samples to each other and the type strains (Refer to Table 6 for codes)

3.4 DISCUSSION

All 24 samples yielded gram positive rods and produced *Listeria*-like colonies on the LSA plates. However, of the 24 isolates only 11 (45.8 %) were phenotypically (BIOLOG and biochemical tests) and genotypically (PCR) *Listeria* (Table 6). Thus, although black colonies on LSA can be used as a presumptive indicator of *Listeria*, genotypical or phenotypical tests are necessary to confirm the presence of *Listeria*. The remaining 13 samples were all PCR negative and only 4 produced a BIOLOG pattern that matched sufficiently with the database to be confirmed a *Listeria*. Most of the *Listeria* type strains although related to the cheese cluster (Fig.3), clustered seperately from the cheese samples. This was with the exception of *L.welshemeri* (F15) and *L.innocua* (F11) which clustered within the cheese group. All the PCR positive cheese isolates were grouped together in the main cluster. Most of the PCR negative samples together with the type strain *Jonesia denitrificans* (A1) were outliers clearly separate from the cheese cluster. This confirmed, that although *Jonesia* was initially part of the genus, it varies from typical *Listeria* both genotypically and phenotypically. Only one isolate (G21) was phenotypically related to the main cheese cluster but did not possess the *iap* gene. This indicates that this isolate shares phenotypical characteristerics with *Listeria*. Further investigations of the phenotype of isolate G21 are necessary to identify and determine the phenotypical similarities between this strain and typical *Listeria*.

In blue cheese, natural acidification occurs during the first 20 days with a final pH of 4.6 to 4.9. This plays a critical role in reducing the number of *Listeria* during the first 50 days of ripening . It is suspected that the proteolytic and lipolytic activity of *Penicillium roqueforti* results in free fatty acids which inactivate the growth of *Listeria* during the initial stages of ripening. However, during the later stages of ripening, the pH increases again, allowing growth of surviving bacteria able to grow at the ripening and storage temperatures (*Papageorgiou* and *Marth*, 1988b).

3.5 CONCLUSION

The above results indicate that 45.8% (11 samples out of the 24 tested) of the samples contained *Listeria* that were able to survive the initial stages of ripening. This emphasises the need for effective pasteurisation and the use of hygenic techniques to eliminate the introduction of *Listeria* in the cheese-making process. Further studies are necessary to determine the source of contamination and the ecophysiology of *Listeria* in Blue cheese.

3.6 ACKNOWLEDGEMENTS

We wish to thank Prof.T.Britz for his assistance in compiling and analyzing the Biolog data. This research was supported by a grant 2034282 from the Foundation for Research and Development of South Africa. L. Ehrenreich was supported by an FRD scholarship.

Chapter 4

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The diversity of *Listeria* and *Listeria*-like isolates prevalent in food and water in Cape Town, South Africa

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Since its early description, *Listeria* has been isolated from a variety of sources including soil, water, plant matter and animals. Although most recorded *Listeria* outbreaks have been linked to contaminated food, most of these foods have a previous history of soil and / or water contact but little is known regarding its prevalence in soil and water. In order to study the ecology of *Listeria* in mixed-culture environments, a sensitive, reproducible and inclusive detection method for all *Listeria*, excluding false positives, is vital. The aim of the work reported here was to ascertain the prevalence and diversity of *Listeria* and bacteria that could be taken to be *Listeria* in various foods and in natural and irrigation waters in order to develop a reliable and inclusive detection method. One hundred and eight water samples, 24 cheese samples, 33 raw and processed fish samples, 30 vegetable samples and 10 processed diary products were subjected to an isolation procedure of *Listeria*. All gram positive non-spore-forming rods were subjected to biochemical tests, and to PCR to detect the *iap* gene, PCR to amplify a region of the 16SrRNA gene specific for the genus and clustered using their whole cell protein profiles. The generally accepted *iap* gene PCR failed to detect all true *Listeria* whereas the 16S-based one was reliable in all cases. We obtained a large number of *Listeria*-like strains, some of which are very distant from the currently accepted members of the genus.

4.1 INTRODUCTION

Since its early description, *Listeria* has been isolated from a variety of sources including soil, water, plant matter and animals (*Frances* et al, 1991; *Bernagozzi* et al,1994; *Welshimer*, 1960; *Weis* and *Seeliger*, 1975; *Fenlon*, 1985; *Hird*, 1986). The hypothesis that listeriosis was only a zoonosis, was only modified after the epidemic outbreak of food borne listeriosis from contaminated coleslaw in the Maritime provinces of Canada in 1984 (*Schlech*, 1983; *Farber* and *Peterkin*, 1991). Although *L. seeligeri*, has sporadically caused disease in humans, the principal food borne pathogen involved in sporadic and epidemic outbreaks of listeriosis is *L. monocytogenes (Farber* and *Peterkin*, 1991)

Although most recorded *Listeria* outbreaks have been linked to contaminated food, some of these foods have a previous history of soil and / or water contact (*Schlech*, 1983). The source of contamination of these foods is usually soil or water. In addition, the organism is able to withstand unfavourable temperatures (*Bayles* et al.,1996; *Farber* and *Peterkin*, 1991) and withstand dehydration and high concentrations of salt (*Bochner*, 1984; *Beumer* et al., 1995). Little is known regarding the prevalence of *Listeria* in soil and water and even less regarding the origin and factors affecting the transfer to animal and plant-based foods. All the above emphasizes the need for more knowledge regarding its prevalence, diversity and growth in these environments.

Knowledge about the minimum infective dose of *Listeria* is limited and the outcome of the infection is severe to immunocompromised people. For these reasons there is a 'zero tolerance' of all *Listeria* imposed on food in several countries (*Jurado* et al., 1993; *Czuprynski*, 1994). Thus, it has become crucial to detect *Listeria* and not merely *L.monocytogenes* in food to establish possible sources of contamination.

Various methods including conventional culturing selective techniques, immunological methods, and a variety of PCR methods have been developed for the detection of *Listeria* (*Lovett*, 1988; *Cassidy* and *Brackett*, 1989; *Kämpfer* et al., 1994). Various PCR detection methods targeting regions of the listeriolysin

and other virulence gene have been published, and were designed to be specific for either *L. monocytogenes*, or for all *Listeria* (*Deneer* and *Boychuk*, 1991; *Gray* and *Knoll*, 1995; *Ericsson* et al., 1995; *Simon* et al., 1996). A method for detecting all *Listeria* by amplifying the *iap* gene has also been published (*Bubert* et al., 1992), but the above methods have failed to detect all pathogenic *Listeria*. Therefore newer PCR detection methods have been designed to target phylogenetically conserved regions, such as the 16S rDNA gene (*Lantz* et al., 1994; *Herman* et al., 1995).

The growth requirements of *Listeria* are still poorly understood and selective media for its recovery from environments where it is present in low ratios are not very effective (*Cassidy* and *Brackett*, 1989; *Farber* and *Peterkin*, 1991). Other factors that complicate the detection of all *Listeria* in natural environments include the poor culturability following exposure to sub-lethal stresses, and the ability of various unrelated bacteria to grow in the generally accepted selective media (*Buchanan*, 1990). In order to detect *Listeria* reliably and to study its ecology in mixed-culture environments, a sensitive and reproducible detection method for all *Listeria*, excluding false positives and false negatives, is vital.

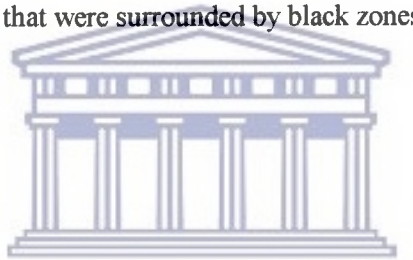
The aim of the work reported here was to ascertain the prevalence and diversity of *Listeria* and bacteria that could be taken to be *Listeria* in various foods and in natural and irrigation waters in order to develop a reliable and inclusive detection method. We report the isolation of a diverse collection of *Listeria* and *Listeria*-like strains, some of which are very distant from the currently accepted members of the genus, and describe a new PCR-method for detection of all *Listeria*.

4.2 MATERIALS AND METHODS

4.2.1 Isolation of strains

One hundred and eight raw water (108) and sewage samples, 24 cheese samples, 33 raw and processed fish samples, 30 vegetable samples and 10 processed dairy products were collected at various points in Cape Town, South Africa, transported to the laboratory on ice and processed within 48hrs.

Twenty-five grams or 25 ml of the sample was pumelled in 225ml of Listeria Enrichment Broth (CM856 + SR142, Oxoid)) in a stomacher for 2 min at medium speed. After 4 and 24 hours incubation at 30°C, 1ml of the overnight enrichment was serially diluted in sterile water, spread-plated onto duplicate plates of Listeria Selective Agar (LSA)(CM854 + SR140, Oxoid) and incubated at 30° C for 48hrs. After 24hrs, 1ml of the primary enrichment was transferred to 4.5ml of 0.5% KOH, vortexed for 1min and spread-plated onto duplicate LSA plates. Colonies that were surrounded by black zones were purified on LSA for further characterization.



4.2.2 Reference strains used

L. monocytogenes ATCC 15313, *L. monocytogenes* NTCC 7973, *L. ivanovii* ATCC 19119, *L. innocua* ATCC 33090, *L. seeligeri* ATCC 35967, *L. welshemeri* ATCC 35897, *L. grayi* ATCC 19120 and *Jonesia denitrificans* ATCC 14870 were utilized as reference strains in all experiments.

4.2.3 Physiological tests for the preliminary allocation to the genus *Listeria*

The morphology and gram reaction of presumptive *Listeria* isolates was determined by using the gram stain and the gram reaction was confirmed with the KOH method (*Powers*, 1995). All gram positive non-spore forming rods were then streaked onto Tryptone Soya Agar (TSA)(CM129, Oxoid) and incubated overnight at 30°C. Biochemical tests were performed on all these samples as well as the type strains. Motility at 20-25°C (0.2% agar), indole production, methyl red test, acetoin production (Voges Proskauer test), citrate utilization according to Simmons, urease production, catalase production, growth at 35°C and fermentation of glucose and the oxygen requirement were performed. Typical *Listeria* are facultative

anaerobes that are motile at 20-25 °C, grow at 35 °C, have a positive methyl red reaction, produce acetoin and catalase (*Seeliger* and *Jones*, 1986). In addition these organisms do not utilize citrate or produce indole, urease or hydrogen sulphide. Isolates that had more than two atypical results were taken not to belong to the genus *Listeria*.

4.2.4 Characterisation of isolates on Biolog GP test panels

All isolates were streaked onto BUGUM (Biolog Inc.,USA) agar supplemented with 0.5 % (v/v) of defribrinilated blood and incubated at 30 °C for 24 hrs. A suspension of the overnight culture was made in 20ml of 0.8% NaCl and inoculated onto the Biolog GP identification test panels according to the manufacturers instructions. After 4 and 24 hrs incubation at 30 °C, the plates were read using a multiscan microplate spectrophotometer at a wavelength of 590nm. The data was analysed using the Microlog software system (Biolog). A dendrogram was created and clustered in relation to its closest relatives.

4.2.5 Detection of the *iap* gene by PCR

The presence of the *iap* gene was determined by the method of *Bubert* et al. (1992). The PCR was performed according to the published protocol except for the following modifications: 0.5U Taq polymerase (Thermoprime, Southern Cross Biotechnologies), 150μM dNTP Mix (Boehringer Mannheim), 1μl of crude bacterial lysate were added to a final volume of 50μl. The DNA amplification conditions were followed according to the protocol, except that the annealing temperature was adjusted to 66 °C. The reaction tubes were overlaid with 20μl of mineral oil and the PCR was performed using a Hybaid Omn-E thermal cycler. The resulting PCR products were separated on a 0.8% agarose gel and visualized by ethidium bromide staining and UV transillumination at 254nm.

4.2.6 PCR of 16S rDNA sequence specific for known *Listeria*

Primers were designed by aligning all known 16S rDNA genes of the genus *Listeria* available at the National Center for Biotechnology Information (NCBI) with those of phylogenetically related bacteria, and

identifying regions conserved for but specific to *Listeria*. The forward primer CLis2 (5' GTTAGAGAAGAACAAGGATA 3') corresponds to position 462 to 482 in *E. coli*, and the reverse primer CLis4 (5' TCTGTCTCCAGAGTGGTCAA 3') corresponds to the complement of the position 1122 to 1142 in *E. coli*. Primers were obtained from Genosys (UK). The template was obtained by suspending one colony in 50 µl of distilled water and incubating at 100 °C for 10 min. The PCR reaction was carried out in a volume of 50μl and contained 50 pmole primer CLis2, 50 pmole primer CLis4, 150μM dNTP mix (Boehringer Mannheim), 3mM $MgCl_2$, 0.5U Taq polymerase (Thermoprime, Southern Cross Biotechnologies) in the reaction buffer supplied with the enzyme, 1μl of crude bacterial lysate, and overlaid with 30μl of sterile mineral oil. After an initial denaturation step at 95°C for 1 min, 25 cycles of 95°C for 1 min, 51°C for 1 min and 72°C for 5 min, were performed, followed by a final extension step at 72°C for 5 min. The PCR reactions were performed on a Hybaid Omni-E thermal cycler. The resulting PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining and UV transillumination at 254nm.

4.2.7 Polyacrylamide gel electrophoresis of whole-cell proteins

All the type strains as well as the 100 food and environmental water isolates were grown on TSA (Oxoid, CM129) at 30°C for 48hrs. Whole-cell protein extracts were prepared as described by *Dagutat* (1990). The modified version *(Kiredjian* et al., 1986) of SDS-PAGE as initially described by *Laemmli* (1970), was performed. All gels were run in a Biorad ProteanII dual cooled vertical slab unit. Gel were scanned with a Hoefer GS300 transmittance/reflectance scanning densitometer at a speed of 13cm/min. The data obtained were analysed with the GelCompar 4.0 programme (Applied Maths, Kortrijk, Belgium). This programme then calculates the Pearson product moment correlation coefficient (r) between the strains, and clusters them using the unweighted pair group method of arithmetic averages (UPGMA). The reference strain, *Psychrobacter immobilis* LMG 1125 was used to determine the reproducibility of electrophoresis by comparing it with the standard protein profile of this organism in the GelCompar 4.0 programme. A correlation (r) of 94% between gels was presumed acceptable for reproducible gels.

4.3 RESULTS

4.3.1 Presumptive identification of *Listeria* using the gram stain, KOH reaction and colony morphology on LSA

One-hundred and twelve isolates were obtained from various sources and were found to be gram positive non-sporing rods that hydrolysed aesculin. Although some isolates were coccobacilli, these were also included since some *Listeria* display coccobacillus morphology (*Schlech* et al., 1983). Some aesculin-hydrolysing rods displayed square-ended *Bacillus*-like morphology, although the colony-morphology was similar to that of *Listeria*. All rod-shaped aesculin producing isolates were further tested using phenotypical and genotypical tests.

4.3.2 Phenotypical evaluation of *Listeria* using classical biochemical tests and Biolog test panels

The biochemical tests were evaluated on the basis that nine out of the eleven tests, matched biochemical profiles of the genus. Most of the biochemical tests for the *Listeria* group matched the biochemical profiles for the reference strains whereas the *Listeria*-like group differed by more than two tests. The commercial kit (Biolog test panels) was used as an additional phenotypical test for the identification of *Listeria* to the species level. A pilot study using the Biolog system on the reference strains revealed that this system could be used to identify *Listeria* to the genus level but not the species level.

4.3.3 Genotypical analysis using the *iap* gene and the 16S rDNA gene

Non-specific binding of the *iap* gene primer set with *E. coli* and *B.subtilis* resulted in the necessity to optimise the PCR conditions for this primer set as described. After optimization, *Listeria* type strains and all the isolates that contained the *iap* gene, resulted in a PCR product of 1500bp (Fig. 8). It was found that the *iap* gene PCR resulted in 8% false positives and 19% false negatives. The reason for using the *iap* primer set was because it was shown to be inclusive of all *Listeria*. However, the high percentage of false negatives in this study, necessitated the development of a more efficient primer set, thus, the development of the 16S rDNA primer set.

The 16S rDNA based PCR was positive for all the *Listeria* type strains but negative for *J.denitrificans*,

E.coli and *B.subtilis*. All the positive isolates resulted in a 680bp product (Fig. 7). Using the partial 16S rDNA gene resulted in 10% false positives and 11% false negatives.

4.3.4 The analysis of whole-cell proteins

Analysis of the whole-cell proteins resulted in the separation of the isolates into two divisions (designated Division A and Division B)(Fig.4). The first division contained all presumptive *Listeria* while the second contained *Listeria*-like bacteria. *Listeria*-like bacteria were defined as gram positive, non-sporing rods that could hydrolyse aesculin but were not classified as *Listeria* according to biochemical tests, whole-cell protein analysis or PCR. Whole-cell protein analysis classified 7% of the vegetable isolates, 72% of the raw or sewage water, 48% of the cheese isolates and all the seafood isolates as belonging to the genus *Listeria*. Division A was further divided into three clusters. Cluster I contained the majority of type strains as well as dairy, seafood and water isolates. All isolates in Cluster I appeared to be closely related to the type strains, consisting predominantly of processed foods (ie. dairy and seafood) and containing three water isolates (1303, 8502 and 9802). This cluster was further subdivided into Group Ia (dairy isolates) as well as Group 1b which contained the majority of seafood isolates as well as the type strains; *L.ivanovii*, *L.seeligeri*, *L.welshimeri* and *L.monocytogenes* (lab strain). Group 1c contained the rest of the *L.monocytogenes* strains as well as *L.innocua*. Isolate 1303 correlated at a level of 0.9 (90%) with *L.monocytogenes* and 8502 clustered well with the type strain *L.innocua*. Cluster II consisted predominately of water samples as well as two vegetable (lettuce) samples (2La and 2Lb). This cluster can further be subdivided into Group IIa which contains water samples, Group IIb contained the vegetable isolates and Group IIc the rest of the water samples. Division A also contained isolates that were separate from the two clusters but still belonging to the division containing the presumptive *Listeria* isolates. This cluster (designated cluster III), contained the remaining seafood samples (S8,S11, S21 and S29) as well as the type strain, *L.grayi*

Division B contained the majority of vegetable samples and some of the dairy isolates. In this division the isolates were mainly unrelated but there were a few isolated clusters. This division, however, contained the water isolate 9702 which was positive for all the tests except for the whole cell proteins analysis. The

whole cell-protein analysis correlated well with the biochemical tests.

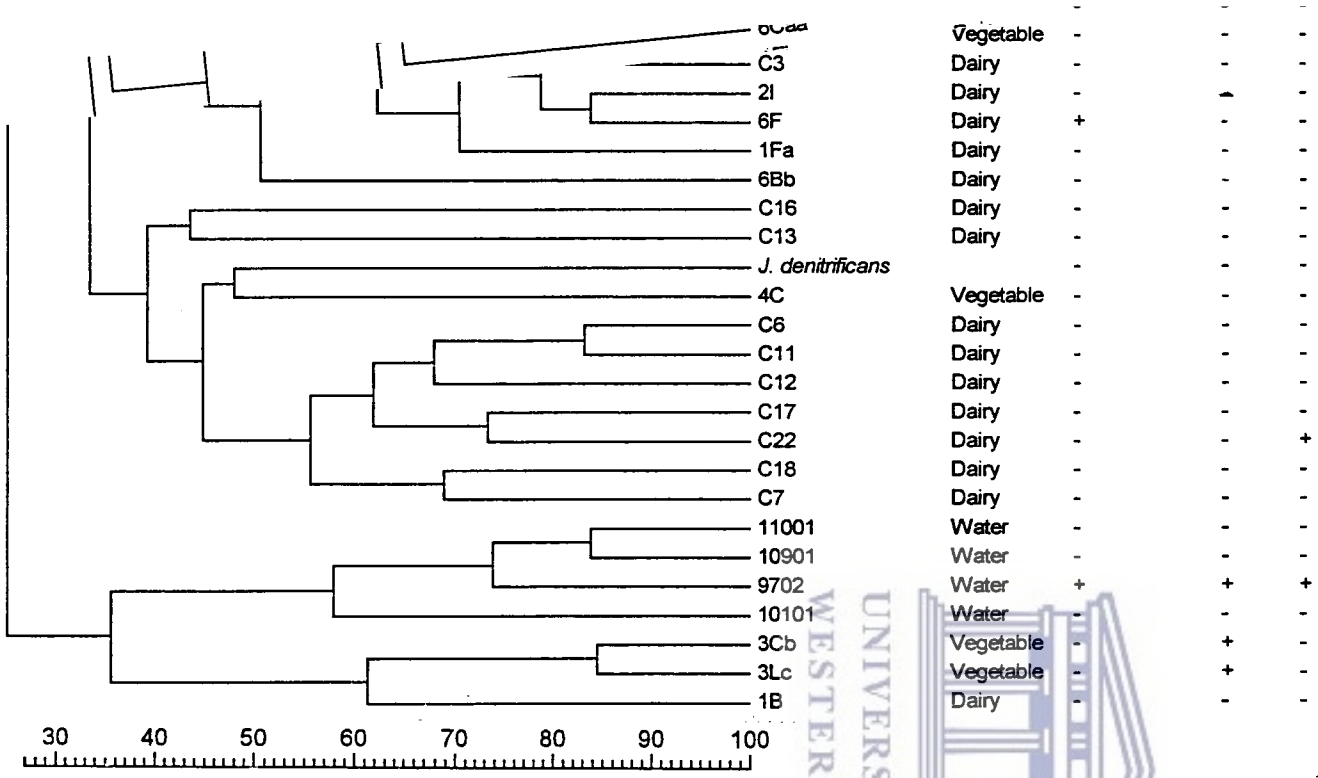


Fig 4. Numerical analysis of the standardized and normalized whole-cell protein profiles of ATCC type strains, water isolates, dairy isolates, seafood isolates and vegetable isolates compared with biochemical tests as well as genotypical results based on the *iap* gene and the partial sequence of the 16SrRNA gene specific for *Listeria.*

4.4 DISCUSSION

The gram reaction, morphology and aesculin hydrolysis, were all criteria used for the initial screening of the isolates. Out of the initial number of isolates, 112 met these criteria. Whereas, 57% of the *Listeria*-like colonies obtained on LSA were confirmed *Listeria*, 43% were not, highlighting the importance of performing further phenotypical and genotypical tests to confirm the isolation of *Listeria*. Oxford formulation (LSA) contains aesculin, ammonium ferric citrate, acriflavine, nalidixic acid and cycloheximide. *L.monocytogenes* has the ability to hydrolyze aesculin. The product of this reaction and ammonium ferric citrate react to form the black halo's around the colonies. Acriflavine is used to inhibit gram positive cocci and gram negatives while nalidixic acid is used as a selective agent for gram positive bacteria in the presence of fungi and gram negative bacteria (*Buchanan*, 1990) . There are many genera which contain gram positive rods that are able to hydrolyse aesculin (*Seeliger* and *Jones*, 1986). Therefore, not all "black colonies" on LSA belong to the *Listeria* (Fig.5). Thus, a better system should be added or new media developed to identify presumptive *Listeria*.

The results of the biochemical tests correlated well with the results of the whole-cell protein profiles (Fig. 4). However, amongst our isolates key tests such as the gram reaction, morphology, tumbling motility between 20 to 25 °C, positive catalase reaction, acid from glucose and no hydrogen sulphide production were among the tests needed for the preliminary identification of *Listeria*. We therefore propose that the biochemical tests be reduced from eleven to six for the preliminary isolation of *Listeria*. In this study, no catalase negative isolates were found although *Bubert* et al. (1997) isolated some catalase negative *L.monocytogenes*.

Standardized protein pattern were initially used for the classification of gram negative bacteria according to their species level (*Kerstens* and *De Ley , 1975; Kämpfer et al.*,1994). However, soon after gram positive bacteria for example the lactic acid bacteria followed (*Dicks* et al., 1987). Earlier investigations of using standardized proteins to differentiate between *Listeria* species revealed no significant differences *(Lamont* et al., 1986) However, our results (Fig. 6) indicate that there are variations in the whole-cell proteins profiles between *Listeria* species as well as within strains in the same species. *Botha* (1996) also observed these differences within type strains of the same species of lactic acid bacteria even though the mean level

of similarity between gels was 94%. This could possibly be a phenomenon unique to gram positive bacteria. *Kämpfer et al.* (1994) hypothesised that the variability in protein profiles could be due to the extraction process used as gram positive cells walls are more resiliant and do not lyse as easily as gram negative cell walls. However, our results including both light and dark bands which could possibly be a phenomonon unique to *Listeria*.

L.grayi is distinctly distant from other *Listeria* (correlation of 63%) while Group IIa in Cluster II has a correlation of 72% relatedness to the cluster containing the type strains and typical *Listeria*. Since this cluster is related to the type strains at a correlation level higher than *L.grayi* but did not contain any type strains, the question whether these isolates belong to a new species or subspecies should be investigated. The clustering of the two vegetable samples (2La and 2Lb) in Cluster II could possibly be because the vegetables were contaminated by soil or irrigated by contaminated water.

Division B contains predominately dairy and vegetable isolates that do not belong to *Listeria* but are also not related to *Jonesia* or each other. Both soft cheeses and vegetables have been implicated in outbreaks of listeriosis. However, according to the results given above, the majority of our vegetable isolates and some of the cheese isolates are *Listeria*-like but not true *Listeria*. This division consists of a diverse collection of genera or species which were unrelated to *Listeria* (correlation between 55 and 30%) and unrelated to each other except for isolated clusterings of water, vegetables and dairy isolates.

The fact that the majority of water, seafood and dairy isolates cluster into essentially three separate groups indicates that presumptive *Listeria* have similar phenotypical characteristics while *Listeria*-like isolates are less structured in there arrangements. Thus, the taxonomy of this group of *Listeria*-like bacteria must be investigated.

4.5 ACKNOWLEDGEMENTS

This research was supported by grant 2034282 from the Foundation for Research Development of South Africa (FRD) to VSB. LE was supported by a FRD scholarship.

4.6 SUPPORTING EVIDENCE

Fig. 5. Photograph of aesculin hydrolysis by *Listeria* type strains and *Jonesia denitrificans* grown on *Listeria* selective agar plates. *L. monocytogenes* ATCC 15313 (a), *L. monocytogenes* NCTC 7973 (b), *L.ivanovii* ATCC 19119 (c), *L. innocua* ATCC 33090 (d), *L. grayi* ATCC 19120 (e), *L. welshimeri* ATCC 35897 (f), *L. seeligeri* ATCC 35967 (g) and *J. denitrificans* ATCC 14870 (h).

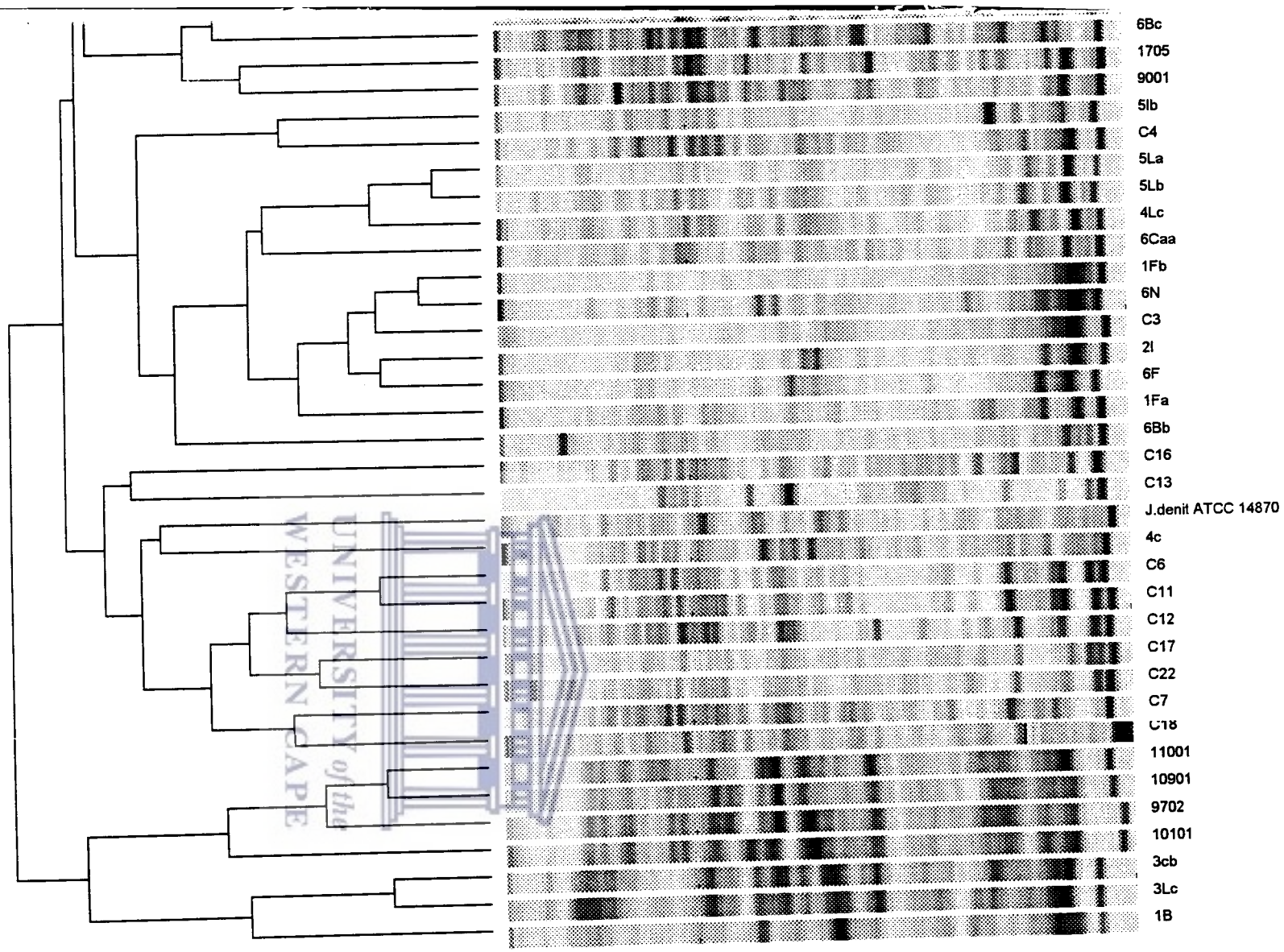


Fig. 6. Numerical analysis of standardized and normalized whole-cell protein profiles of the *Listeria* type strains, *J.denitrificans* and food and water isolates.

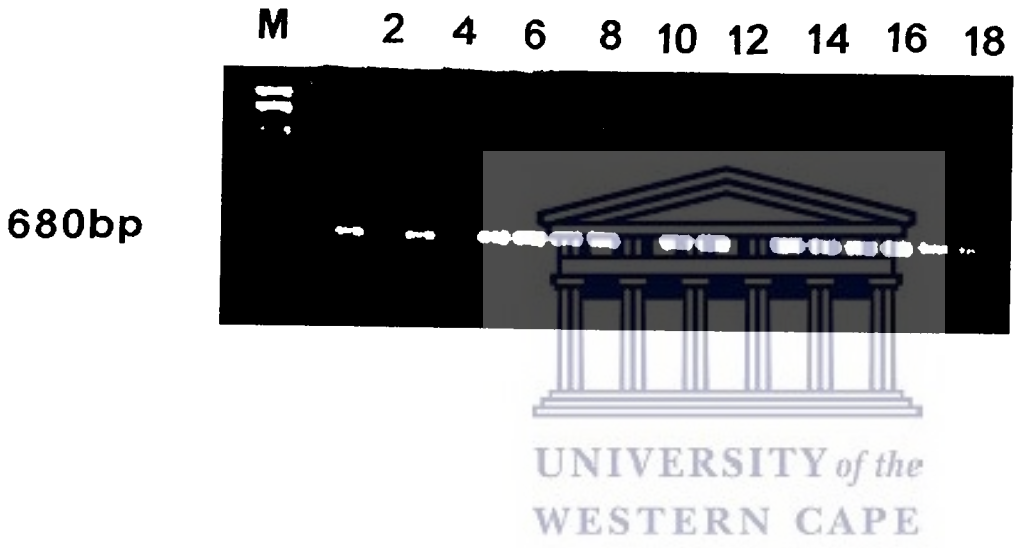


Fig. 7. Agarose gel electrophoresis of products from PCR amplification using primers directed against the 16S rDNA gene. Lanes () containing PCR products of the some environmental water isolates: λHind III marker (M), *L.monocytogenes* ATCC 15313 (1), *J. denitrificans* ATCC 14870 (2), *L. monocytogenes* NCTC 7973 (3), blank (4), 1104 (5), 1303 (6), 1502 (7), 1604 (8), 1705 (9), 2303 (10), 2901 (11), 3002 (12), 5104 (13), 7304 (14), 8001 (15), 8201 (16), 8402 (17) and 9402 (18)

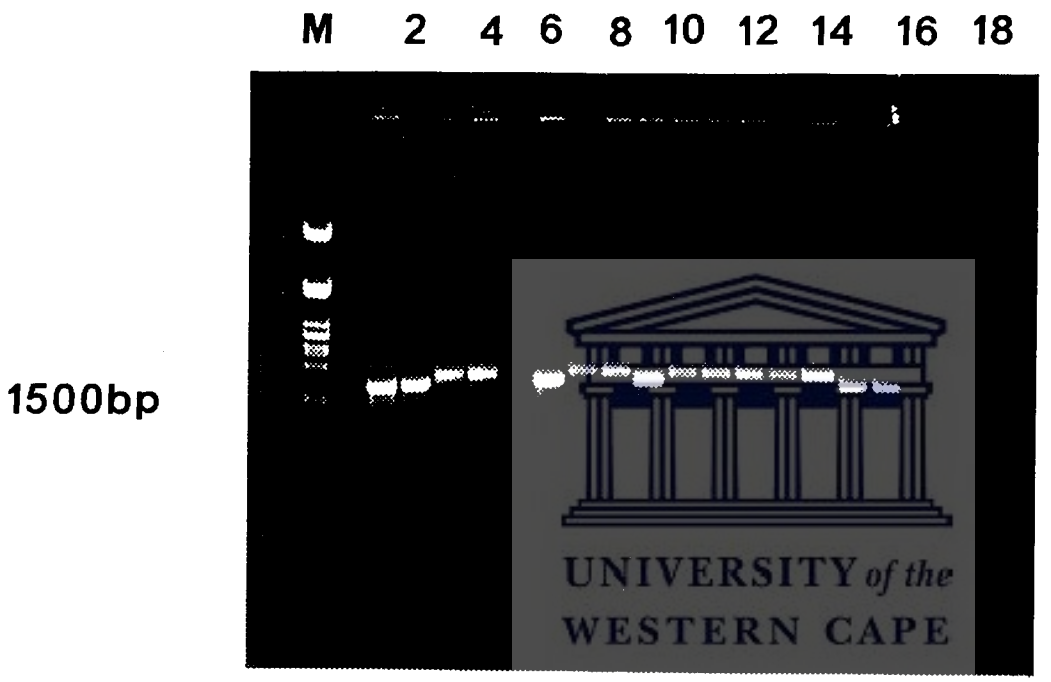


Fig. 8. Agarose gel electrophoresis of products from PCR amplification using primers directed against the *iap* gene. Lanes () containing PCR products of the some environmental water isolates: λHind III (M), *L. monocytogenes* ATCC 15313 (1), *L. monocytogenes* NCTC 7973 (2), 1104 (3), 1303 (4), *J. denitrificans* ATCC 14870 (5), 1502 (6), 1604 (7), 2303 (8), 3002 (9), 5104 (10), 7304 (11), 8001 (12), 8201 (13), blank (14), 8402 (15), 9502 (16) and 9702 (17).

Chapter 5

Conclusions

The results show that *Listeria* and *Listeria*-like organisms are regular contaminants of water, seafood, dairy products and vegetables.

- Dairy products, especially soft-cheeses have been implicated in epidemic listeriosis outbreaks. The results show that soft-cheeses were frequently contaminated by *Listeria* and that very few dairy products were contaminated by *Listeria*-like organisms.

- The regular isolation of *Listeria* from water samples indicate that more research should be done on this river since it is used as a recreational swimming facility by the children from surrounding communities.

- Seafoods, including mussels and oysters are regularly contaminated by *Listeria*.

- Dairy, water and seafood isolates clustered together in specific niches.

- A cluster of water isolates were *Listeria*, not closely related to the type strains but more closely related than *L.grayi*. We pose the question whether these isolates belong to a new species or subspecies.

- The majority of vegetables were contaminated by *Listeria*-like organism rather than true Listeria and there was no significant link between water and vegetable samples.

- A large majority of presumptive *Listeria* were actually *Listeria*-like and not true *Listeria*. Therefore methods for the preliminary isolation of *Listeria* should be improved.

- At present eleven biochemical tests are used for the preliminary identification of *Listeria*. We propose that these tests be reduced to six which includes, gram reaction (KOH), morphology (simple stain), motility at 20-25 °C, catalase product , no hydrogen sulphide and acid from glucose.

- The 16SrRNA gene and *iap* gene primer set were able to detect most but not all *Listeria*.

Chapter 6

Author's perspective

Working on *Listeria* has resulted in more questions than answers. The biggest problems being that certain basic characteristics about this organism is lacking. These include the taxonomic position of species within the genus, the infective dose and the mode of infection. Some other specific question that still need to be answered are listed below :.

1) The tendency has been to concentrate on *L.monocytogenes* since this is the primary human pathogen. However, by excluding all other species have we not created a limited view of the organism as it interacts with other species and genera under "normal" conditions? What effect does this interaction have on *Listeria*?

2) The water isolates were related, yet phenotypically distinct from the *Listeria* type strains. What are the reasons for both the similarities and the differences between the type strains and these water isolates?

3) The genotypical methods used in this study identified a high proportion of *Listeria* from a variety of sources. However, will this method be able to detect 1 organism/25g which is the "zero-tolerance" standard currently imposed.

4) Aesculin hydrolysis which has been widely used as a presumptive indicator of *Listeria*, is not very accurate since other genera can also hydrolyse aesculin. How can this system of inhibition and selection be improved to exclude *Listeria* -like organisms?

5) In nature, how does *Listeria* compete against more predominant pathogens such as *Salmonella*? Which conditions allow *Listeria* to outcompete these bacteria and cause disease? Also with the

suppression of predominant competitors with commonly used antibiotics or environmental stresses will listeriosis epidemics increase?

6) At present the emphasis is on isolating and identifying *Listeria*. However, do the benefits of prevention and education outweigh the economic implications of listeriosis?

7) Other questions that need to be answered include what the minimum infective dose needed for infection is and why some species that are haemolytic and pathogenetic to animals are not human pathogens

8) In South Africa listeriosis is not a reportable disease. Is the disease prevalent in South Africa and what is the mortality rate? Is this prevalence seasonal or not?

9) Are people in the Western Cape region (due to the high incidence of TB) more likely to suffer from foodborne listeriosis than elsewhere in South Africa? Since many pregnant woman exhibit flu-like symptoms which can be treated by normal flu antibiotics has the disease gone undetected in the Western Cape?

10) What is the niche of *Listeria*? Is it soilborne or part of the normal intestinal flora or is it a saprophyte part of the food cycle?

11) Although *Listeria* is a non-sporing organism it is extremely tolerant to various environmental stresses. What mechanisms play a role in this tolerance? Is thermotolerence related to intracellular position of the pathogen or not? As for psychrotolence, what role does the cold-stress proteins play?

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Summary

Listeria are gram positive non-sporing rods that were found in diverse environments including water, seafood, dairy products and vegetables. Outbreaks of listeriosis due to the consumption of foods contaminated with *L.monocytogenes*, coupled with the high mortality rate and the risk posed to immunocompromised individuals have resulted in more focus being placed on *Listeria*-free products. Although most recorded *Listeria* outbreaks have been linked to contaminated food, most of these foods have a previous history of soil and / or water contact but little is known regarding its prevalence in soil and water. The aim of the work reported here was to ascertain the prevalence and diversity of *Listeria* and bacteria that could be regarded as *Listeria* in various foods and in natural and irrigation waters in order to develop a reliable and inclusive detection method. Various methods including conventional culturing selective techniques, immunological methods and a variety of polymerase chain reaction methods have been developed for the detection of *Listeria*. In this study, preliminary physiological tests were utilized to detect *Listeria* from 108 natural and irrigation water samples, 34 dairy samples, 33 raw and processed fish samples and 30 vegetable samples. After these preliminary tests, one-hundred and twelve isolates were found to be gram positive non-sporing rods that could hydrolyse aesculin. These isolates were further tested using both genotypical (PCR with primers directed at the 16S rRNA gene and the invasive associated protein gene) and phenotypical (BIOLOG and whole-cell protein analysis). The whole protein profiles divided the various isolates into two main divisions namely, *Listeria* and *Listeria*-like organisms. A diverse selection of *Listeria* and *Listeria*-like strains, some of which are very distant from currently accepted members of the genus, were isolated. Dairy products (especially soft cheeses), seafoods and water isolates clustered in separate niches within the Listeria division. The majority of vegetables, however, were mainly contaminated by *Listeria*-like organisms and not true *Listeria*. The presence of these *Listeria* in a local river was of some concern since the local children used the river as a swimming facility. Some isolates from the river, although related to the type strains, were separate from typical *Listeria*. Thus, the possibility exists that this group could be a new species or subspecies. The development of a 16S rDNA primer set (CLis2 and CLis4) for the detection is reported. All isolates that contained the invasive associated protein (*iap*) gene resulted in a PCR product of 1.5kb and those that contained the 16S rRNA

gene resulted in a product of 0.68kb. Although these primer sets could detect most *Listeria* , they could not detect all. The development of more selective media for the preliminary detection of *Listeria* and the reduction in the number of physiological tests, were recommended as well as the optimisation of the 16S rRNA PCR for the direct detection of *Listeria* in food products.