DEVELOPING AN INTEGRATED PHAGE-PCR ASSAY FOR RAPID DETECTION OF *LISTERIA MONOCYTOGENES* IN FOODS

By

MOHAMED M. EL EMAM

This thesis is submitted as a partial fulfilment for the degree of

Doctor of Philosophy

The University of Nottingham

Faculty of Sciences

United Kingdom

June, 2012

ABSTRACT

Listeria monocytogenes is a common food borne pathogen which is an important contaminant found in various food factory environments, because of its ability to survive in a wide range of environmental conditions, and to grow at refrigeration temperatures. *L. monocytogenes* has caused both occasional outbreaks and sporadic cases of food-borne illness characterised by high mortality rates. In the UK and other European countries, there has been a conspicuous rise in the number of reported cases of "Listeriosis" recently. Hence, development of efficient and rapid methods for detection of this microorganism in various foods is of great significance for the food industry; and is needed to ensure the safety of foods that are considered at high risk of contamination. Conventional bacteriological methods (e.g. ISO 11290-1/A1) for the detection and quantification of *L. monocytogenes* are laborious and time consuming. Therefore, development of a rapid and reliable test capable of detecting very low numbers of the organism in ready-to-eat products is required. To address this, a phage amplification assay has been developed as a rapid method for the detection of *L. monocytogenes* using the broad host range phage A511.

Successful development of the assay required identification of a virucide that could achieve inactivation of the phage without affecting the viability of the target cell to be detected. Several different substances were evaluated as potential virucides, and among the tested materials, tea infusions were found to be the most effective virucidal agent for this experiment. The efficacy of the

new assay was tested using Stilton cheese, as a representative high risk dairy product, and a method was developed to use centrifugation to concentrate bacterial cells present in samples of half-Fraser broth enrichments. The cells were detected by using the new phage amplification assay and this combination of techniques was shown to be able to detect low numbers of cells in shorter times than can be achieved using conventional culture methods.

An additional molecular identification step was also developed so that the identity of the cells detected could be confirmed using a multiplex PCR which targeted conserved regions of the *Listeria* 16S rDNA genes. In this assay, two amplified DNA fragments were generated confirming the presence of *Listeria* genus (400 bp band) and also *L. monocytogenes* species (287 bp band). An advantage of this combined phage-PCR method its ability to detect only viable cells in food samples. The combined assay was then tested on a wide range of spiked food samples, including Camembert cheese, pasteurised milk, minced meat, turkey meat and smoked salmon. The obtained results showed that the limit of detection was as low as 20 (± 5) cfu per 25 g, and duration needed for the detection and molecular conformation of speciation was 2 days (44 h), compared to 5 days using conventional culture methods.

The combined phage-PCR assay was able to achieve a sensitive and specific identification of viable *L. monocytogenes* present in foods within 48 h, and therefore would allow for rapid screening of food products prior to release from the factory.

ACKNOWLEDGEMENTS

Praise always to almighty “ALLAH”, the most merciful, the compassionate for giving me belief, health, confidence, strength, and blessing to accomplish this thesis as a partial fulfillment of the requirements for the degree of doctor of philosophy.

After all those years, first and foremost I would like to express my sincere gratitude to Allah for giving me strong faith and wonderful moments in my time and provided me the blessing to complete this work.

I would like express my utmost appreciation to my supervisor Dr. Cath Rees for giving me the opportunity to run this project, and also for her guidance, support, kindness and patience encouragement, and for her availability at any time. I do not think without her genius strategy I would get this far for this thesis.

Thank you, Dr. Cath!

A Special appreciation goes to my lab assistant Wendy and Lorraine for her help with preparing media and always with a smile. I would also like to thank a chief technician Dave who helped me to get any equipments, materials and for so much problem solving along the way. Furthermore, my sincere pleasure to all my colleagues at the Microbiology laboratory (Sutton Bonington Campus) for the excellent lab environment especially Omar Elhenshir, Ogi, Ben, Hassan, and Lawrence. I feel lucky to work with you during my study.

I also want to thank the Libyan government; Biotechnology Research Center for the financial support throughout my PhD program.

Finally, yet importantly, I thank my own family; wife, son, daughters, Mother, brothers and Mother-in- law who have always for cheering me up, supported and encouraged me and also for their love and prayers for me. Indeed, your love and support are the most important things in my life!

Table of Contents

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 History of *Listeria monocytogenes*

Listeria monocytogenes is an opportunistic food-borne pathogen which causes very serious diseases to humans. *L. monocytogenes* is a bacterium that is frequently overlooked as a probable cause of infection due to its particular growth and infection characteristics (Aarts *et al.,* 1999; Bortolussi *et al*., 2008). After infection, there is usually a long incubation period (e.g. 1 to 10 weeks), which makes source tracking by typical epidemiological approaches difficult (Chen *et al.*, 2005). This bacterium has a temperature growth range between 1 °C and 45 °C under both aerobic and facultative anaerobic conditions (Jemmi and Stephan, 2006). *L. monocytogenes* is commonly found in refrigerated food stuffs, both processed and unprocessed, and its ability to grow in diverse food environments has been one of the many challenges presented by this hazardous bacterium.

The bacterium was first reported in 1891 in human tissue samples from patients in Germany (Gray and Killinger, 1966). In 1911, a Swedish researcher by the name of Hulpher isolated what was probably *L. monocytogenes* from a rabbit with miliary necrosis of liver (Juntila, 1988). Over a decade later Murray *et al.* (1926) in the United Kingdom isolated it from infected rabbits and guinea pigs, described the organism and named it *Bacterium monocytogenes* (Farber and Peterkin, 1991). In the following year (1927), a similar organism was isolated from the liver of infected gerbils (*Tatera lobengulae*) in South Africa by Pirie

who named it *Listerella hepatolytica* (Farber and Peterkin, 1991). Two years later (in 1929), the first human case of Listeriosis was reported (Farber and Peterkin, 1991), moreover the first perinatal case was reported in 1936 (Buchanan and Roland, 2000). In 1940 the name of the organism was finally resolved and *Listeria* was proposed as a name for the genus with *Listeria monocytogenes* as the type species (Pirie, 1940).

In addition to human disease, where the manifestations of infection include abortion in pregnant women, meningitis, intra-uterine infection and septicemia (Coffey *et al*., 1989; Hird and Genigeorgis, 1990), *L. monocytogenes* has been reported to cause various diseases in a wide range of domestic animals, and has been associated with invasive infections of more than 40 species of mammals and birds. As well as this the bacterium has also been isolated from amphibians, fish, crustaceans, insects and reptiles (Hird and Genigeorgis, 1990; McCarthy, 1990; Ryser and Marth, 1999).

The mode of transmission for *L. monocytogenes* has been shown to occur through many routes including from mother to child (vertical), direct animal contact (zoonotic), hospital acquired (nosocomial), and food-borne transmission (Buchanan and Roland, 2000). Historically zoonotic infection was mostly reported with infection associated with those who worked with dairy cattle, in particular associated with spontaneous abortions in dairy maids. Vertical transmission may also be significant today, but it is often not reported since the cause of many early stage spontaneous abortions are not investigated. Thus food-borne transmission is considered to be the most important route for human listeriosis disease transmission. The importance of *L. monocytogenes* as

a food-borne pathogen has become increasingly evident since the outbreaks reported in North America since 1981.

In the 1980s there were several outbreaks reported in both the United States and Europe and it was fully recognised that *L. monocytogenes* contaminated food, which had most likely been consumed without prior cooking, was the primary source of infection (Broome *et al*., 1990; Bayles *et al.*, 1996). Since then the organism has been found in a wide range of food samples including milk and other dairy products, meat, salad and cheese (Berrada *et al*., 2006; Little *et al.*, 2009; Poltronieri *et al*., 2009), and the World Health Organisation considers that the primary mechanism of transmission of this pathogen to humans is through various foodstuffs contaminated during production and processing (Sorinano *et al.*, 2001). Indeed, of all human infections, 99 % of the outbreaks appear to be food-borne (Ivanek *et al*., 2006).

1.2 Infections with *Listeria monocytogenes*

Since the first well documented food-borne outbreaks of listeriosis occurred, a world-wide concern about the presence of *L. monocytogenes* in food stuff has been generated. Even small numbers of *L. monocytogenes* can grow to large numbers in foods stored at low temperatures, and ultimately they may become a serious source of infection risk. As this organism is ubiquitous and able to grow at refrigeration temperatures (Farber and Peterkin, 1991), cold-stored foods with long shelf-display times that are eaten without further heat treatment pose a high risk of causing *Listeria* infection.

In adults the primary manifestations of listeriosis include meningitis and septicaemia, with the highest risk of listeriosis again being immunocompromised individuals, such as recipients of organ transplants who are required to take immune-suppressive drugs (Patel and Paya, 1997). However it has also been reported that virulent strains can cause gastroenteritis in healthy individuals (Drevets and Bronze, 2008). In pregnant women infection can occur at any stage of pregnancy and may manifest itself only as flu-like symptoms in the mother, but this can be followed by abortion or stillbirth of newborns; the outcome of infection of neonates is dependent on the time of infection and clinical signs (Schuchat *et al*., 1991). Listeriosis in children older than thirty days is very rare, except in those with underlying disease.

The transmission and pathogenesis of listeriosis generally depends on the ability of the *L. monocytogenes* to survive and to transfer to humans from their original sources through food products (Roberts *et al*., 2003) and therefore those genes which help it survive various environmental stresses contribute to its overall virulence. However, apart from this, the virulence of different strains of the human pathogen *L. monocytogenes* has been correlated to several specific antigenic variations. *L. monocytogenes* has been grouped into thirteen serotypes, but only three of them (e.g. 1/2a, 1/2b and 4b) are responsible for more than 95 % of the reported human listeriosis cases, with the majority of the reported cases attributed to serotype 4b strains (Abdelgadir *et al*., 2009; Bosilevac *et al*., 2009).

Some of the most serious outbreaks of listeriosis occurred in 1980s with several well-documented outbreaks recorded after consumption of contaminated Mexican style cheese, coleslaw, and Vacherin Mont d'Or soft ripened cheese (Norton *et al*., 2001). In addition gravad and cold smoked rainbow trout are examples of uncooked products that have been reported to cause outbreaks of listeriosis (Ericsson *et al*., 1997a) however, outbreaks of listeriosis are most commonly associated with foods, such as soft cheeses, hot dogs, processed meats and salami, paté, pasteurised milk products, when there has been a failure in pasteuriser or post-process contamination (e.g. chocolate milk and butter). It can also be found due to natural contamination in unpasteurised milk, fish products such as cooked shrimp, smoked salmon, and tuna, salad and raw vegetables, in particular coleslaw (Buchanan and Roland, 2000). Many studies have established a high prevalence of *L. monocytogenes* in a variety of food processing environments and these types of ready-to-eat foods (Kathariou 2002; Ramaswawy *et al*., 2007).

One of the challenges for characterising outbreaks associated with food-borne listeriosis is that the relatively long time periods it takes from the time of consuming foods contaminated with *L. monocytogenes* and the onset of listeriosis, which, in most cases, makes it difficult to trace sources of these outbreaks (Farber and Peterkin, 1991). Outbreaks with a common source are quite easily identified, for instance Aureli *et al.* (2000) described investigation of an outbreak of febrile gastrointestinal illness that occurred in over 1500 students. Corn and tuna salad that were contaminated with *L. monocytogenes* served in a school cafeteria appeared to be the source of this outbreak. On the

other hand, when outbreaks are associated with food produced by a company that widely distributes its products it becomes more difficult to identify. For instance a large multistate outbreak of listeriosis in the United States was shown to be due to consumption of contaminated hot dogs that affected a total of 101 individuals with 21 % mortality rate (Ramaswawy *et al*., 2007) and this was first identified by the national *Listeria* surveillance service rather than by local physicians (Anon, 1999).

1.3 Most significant outbreaks of Listeriosis by region

A wide range of food products have been shown to be associated with both outbreaks and sporadic cases (Table 1.1) and have resulted in a number of outbreaks across the world. In 2008, 58 cases of listeriosis occurred in Canada associated with an outbreak of *L. monocytogenes* because of consuming Deli meats and 20 of the cases resulted in deaths (Todd and Notermans, 2011). In the UK 1987–1989, a wider listeriosis outbreak occurred, with 355 persons becoming infected with *L. monocytogenes* by eating contaminated paté with 94 of the cases resulted in deaths (McLauchlin *et al*., 2004).

Table 1.1: Food-borne outbreaks of human listeriosis

Region	Country	Date	Food Implicated	Cases (deaths)	Serotype
Australasia	Australia	1991	Smoked mussels	4(0)	1/2a
	Australia	2005	Deli meats	2 (2)	-
	New Zealand	1980	Shell or raw fish	22 (7)	1/2a
Europe	Czech Rep.	2006	Soft cheese	78 (13)	-
	Finland	1998–1999	Butter	25 (6)	3a
	France	1993	Pork tongue in aspic	279	4b
	France	1999–2000	Pork rillettes	10(2)	4b
	France	1999–2000	Pork tongue in jelly	32 (10)	4b
	Germany	2006	Harz cheese	6 (1)	-
	Italy	1997	Sweet corn salad	1566 (0)	4b
	Norway	2007	Raw milk soft cheese	21(5)	-
	Spain	2005	Unspecified meat	2 (0)	-
	Sweden	1994–1995	Cold-smoked rainbow trout	9(2)	4b
	Switzerland	1983–1987	Soft cheese	122(34)	4b
	UK	1987–1989	Pate	355(94)	4b and 4 not 4b
	UK	1988	Soft cheese	-	-
	UK	2003	Sandwich	4 (?)	1/2
North America	USA	1976	Raw salad	20 (5)	4b
	USA	1994	pasteurized chocolate milk	45(0)	1/2b
	USA	2000	Turkey meat	29 (7)	-
	USA	2002	ready-to-eat meats	54 (8)	4b
	USA	2003	Mexican-style cheese	12 (?)	4b
	USA	2007	pasteurized milk	5(?)	-
	Canada	1981	Coleslaw	41(18)	4b
	Canada	2002	Cheese	17 (0)	4b
	Canada	2008	Deli meats	58 (20)	-
	Canada	2008	Raw milk soft cheese	21 (1)	-

Adapted and updated from McLauchlin et al., 2004; Dawson et al., 2006; CDC, 2007; Swaminathan and Gerner-Smidt, 2007; Todd and Notermans, 2011.

1.4 Presence of *Listeria monocytogenes* in the Food Processing Environment

The initial source of contamination by *L. monocytogenes* in food processing plants is their presence in raw materials that are brought into the plant (Lawrence and Gilmour, 1995; Berrang *et al*., 2002). Once established, *L. monocytogenes* has been found to survive extremely well in various processing plant environments such as dairy plants, fish processing plants, meat and ready-to-eat product processing plants (Pritchard *et al*., 1995). In addition, they have been found on some equipment surfaces after inadequate cleaning and disinfection, and this can lead to subsequent contamination of foods during processing (Norton *et al*., 2001b).

It has been concluded that by controlling *L. monocytogenes* growth in such environments it is possible to minimize, or even to prevent, hazards of food product contamination during processing and display (Tompkin, 2002). However, the cleaning of surfaces in food-processing facilities to achieve the eradication of *L. monocytogenes* has been found to be difficult due to the ability of *L. monocytogenes* to form biofilms on a variety of food processing surfaces, including glass, plastic, rubber, and stainless steel (Blackman and Frank, 1996; Somers and Wong, 2004). The formation of biofilms may be influenced by several factors, such as physical and chemical properties of the substrate for attachment, temperature, the particular characteristics of the strains, the physiological state of the bacteria and the presence of other microorganisms (Pan *et al*., 2006).

This difficulty in maintaining a pathogen-free environment means there is a need to implement programmes to monitor both environmental levels and the products produced for the presence of *L. monocytogenes.* The National Advisory Committee on Microbiological Criteria for foods requires the implementation of Hazard Analysis Critical Control Point system (HACCP) and process control strategies. There are several strategies recommended to prevent *L. monocytogenes* occurrence in food plants, which include implementation of a sampling program that can be used to assess whether levels of the organisms in the processing environment are under control. If the organism is identified, follow-up sampling is recommended so that the sources can be detected and corrective measures taken. However it is equally important to prevent the initiation and growth of *Listeria* and so strict temperature control of products, particularly in ready-to-eat foods that can support growth of the organism. Generally both short-term and long-term assessments of the sampling results are used to allow early problems and trends to be identified that may indicate a problem in released product (Tompkin, 2002).

1.5 Presence of *Listeria monocytogenes* in Dairy Processing

L. monocytogenes has a significant ability to reside in different places in the environment of dairy plants (Klausner *et al*., 1991; Pritchard *et al*., 1995; Menendez *et al*., 1997). Dairy processing plants in the European Union (EU) countries are obliged to control *L. monocytogenes*, particularly because dairy

products have been associated with many of the reported listeriosis outbreaks in Europe (De Buyser *et al*., 2001; Lunden *et al*., 2004). Indeed the emergence of *L. monocytogenes* as a serious food-borne pathogen dates back to thirty years ago, with different outbreaks of listeriosis being directly linked to consumption of contaminated soft ripened cheeses (Norton *et al*., 2001a).

Classically Listeriosis was associated with consumption of unpasteurised dairy products. In Switzerland, between 1983 and 1987 outbreaks were caused because of unpasteurised soft cheese, and later in France, a similar outbreak occurred in 1995 because of a Brie-type cheese prepared from unpasteurised milk. Therefore, from a risk management point of view, all above mentioned outbreaks involved the consumption of raw milk or soft cheeses made from unpasteurised milk (Goulet *et al*., 1995; Lunden *et al.*, 2004). In an earlier study, Fleming *et al*. (1985) reported that case-control studies in Massachusetts showed that *L. monocytogenes* was isolated from raw milk which was associated with the outbreak occurred. In addition, several outbreaks of listeriosis were reported being related to the consumption of contaminated milk, and have been causing great concern in the dairy industry (Griffiths, 1989).

However dairy products made from pasteurised milk might be contaminated in subsequent stages of production with *L. monocytogenes* and result in outbreaks (Lyytikainen *et al*., 2000). For instance in Finland between 1998 and 1999, the source of an outbreak was found to be contaminated butter. An investigation of the plant showed that the cream pasteurizer was operating correctly but the outbreak strain was detected in environmental samples from

the packing machines, from the screw conveyor of the butter wagon and from 2 floor drains beneath the butter wagon of the packaging line, suggesting that the product was contaminated after the pasteurisation process. In addition to this example, post-pasteurisation contamination of milk or curd within the processing environment has been implicated as the cause of other outbreaks.

1.6 Presence of *Listeria monocytogenes* in Fish Processing

L. monocytogenes has occasionally been isolated from seafood products including ready-to-eat products, e.g. shrimp, rainbow trout, smoked salmon, gravad salmon, crawfish and fish salad (Table 1.2). The bacterium most likely enters fish production plants through sea water and presence on slaughtered fish, and hence leads to contamination of the processing line and finished products (Embarek, 1994; Destro *et al*., 1996; Ericsson *et al*., 1997a; Brett *et al*., 1998; Thimothe *et al*., 2002). The incidence of *L. monocytogenes* in fish processing plants for farmed salmon and trout, and during fish smoking have been a major subject of several studies (Rørvick *et al*., 1995; Rørvick *et al*., 1997; Heinitz and Johnson, 1998; Autio *et al*., 1999). For instance Dillon *et al*. (1992) reported that smoked cod fillets, taken from retail outlets, were found to be highly contaminated with *Listeria.* Despite this only a relatively small number of outbreaks have directly been linked to the consumption of contaminated fish and fish products (Rørvik *et al*., 2000; Chou *et al*., 2006).

In their study Ericsson and Stålhandske (1997) reported an outbreak of listeriosis when vacuum-packaged rainbow trout were consumed. The

contamination was also found in other fishery products, particularly vacuum-packaged cold-smoked fish again implicating post-process contamination caused by production equipment. Moreover Eklund *et al.* (1995) reported that fish was exposed to water which was contaminated with *L. monocytogenes* during transport. Contamination of product ultimately can mean that the bacterium is transferred to slicing machines, tables, cutting surfaces, etc, and causes more risk of contamination of product (Lundén *et al*., 2000).

Even though the processing environment has been always reported as being the major contamination source for the final products (Johansson *et al*., 1999; Dauphin *et al*., 2001; Norton *et al*., 2001; Thimothe *et al*., 2002; Gudmundsdóttir *et al*., 2005), other studies showed that food raw materials were found to be another major source of contamination in the final products in some food factories (Eklund *et al*., 1995; Norton *et al.*, 2001; Gudmundsdóttir *et al*., 2005). Three fish smoking plants in Switzerland were investigated by Jemmi and Keusch (1994). In this case the researchers concluded that raw fish were the most frequently contaminated food products with *Listeria* whereas the finished products were less contaminated. The researchers also reported that the serotype 1/2a was the most frequent *Listeria* serotype isolated from the examined seafood products (Johansson *et al*., 1999; Dauphin *et al*., 2001; Nakamura *et al*., 2004) and since serotype 4b is more commonly associated with human disease a low prevalence of this serotype may explain why the number of reported outbreaks was low.

Table 1.2. Listeriosis outbreak connected with seafood consumption

Suspected seafood	No. of cases (death)	Symptoms	country	Published year
Shellfish and raw fish	22(6)	Premature labour, foetal distress, respiratory symptoms, meningitis, flu-like illness, urinary tract symptoms, diarrhoea, vomiting	New Zealand	1984
Fish	1(0)	Meningitis	Italy	1989
Smoked mussels	3(0)	Diarrhoea, vomiting	Tasmania	1991
Shrimps	11(1)	Fever, nausea, vomiting, musculoskeletal symptoms, diarrhoea, foetal demise	USA	1994
Cold-smoked ”gravad” rainbow trout	8(2)	Aminitis, meningitis, premature birth, fever, septic arthritis, septicaemia	Sweden	1997
Smoked mussels	3(0)	Perinatal lethargy, malaise	New Zealand	1998
Cold-smoked rainbow trout	5(0)	Fever, vomiting, fatigue, arthralgia, headache, nausea	Finland	1999
Imitation crabmeat	2(0)	Diarrhoea, cramps, fever, projectile vomiting	Canada	2000

Lennon et al., 1984; Facinelli et al., 1989; Riedo et al., 1994; Ericsson et al., 1997; Brett et al., 1998; Miettinen et al., 1999; Farber et al., 2000.

1.7 Characteristics of *Listeria monocytogenes*

1.7.1 Morphology

L. monocytogenes is a Gram-positive, non-spore forming, facultatively anaerobic, regular short rod-shaped bacteria that can be pleomorphic (Donnelly and Baigent, 1986; Liu, 2006). They are motile between 20 °C to 25 °C using few peritricious flagella which are only visible after culturing below 30 °C (Seeliger and Jones, 1986), and their motility has a characteristic tumbling form when cells are grown in broth culture. The size of the bacterium is in the range of 0.4 – 0.5 µm in width and 2.0 – 6.0 µm in length (Seeliger and Jones, 1986). Cell isolates of newly cultured *Listeria* possibly appear as very short rods that appear almost coccoid, however filaments of 6 – 20 µm in length also develop, especially in older cultures or when cells are subjected to stress (Donnelly and Nyachuba, 2007).

The colonies appear bluish-to-grey under normal illumination and a characteristic of blue green sheen is produced by obliquely transmitted light (Schuchat *et al*., 1991). *L. monocytogenes* grows well on Brain Heart Infusion agar as well as on Tryptic Soy Agar (Hitchens, 1996) and Nutrient agar medium, producing colonies of about 0.5 - 1.5 mm in diameter after 24 – 48 h incubation (Seeliger and Jones, 1986).

1.7.2 Growth Requirements

Despite the fact that *L. monocytogenes* is found in such a variety of environmental niches, it is a relatively fastidious in its nutritional requirements when grown in the laboratory. In addition to a carbohydrate as energy source

for growth, it needs amino acids such as isoleucine, leucine, cysteine, and the vitamins biotin, riboflavin and thiamine (Premaratne *et al*., 1991; Glaser *et al*., 2001).

The optimum growth of *L. monocytogenes* occurs at a temperature range between 30 °C and 37 °C (Seeliger and Jones, 1986). However *L. monocytogenes* is psychrotropic in nature and can grow over a wide range of temperatures (e.g. from below 1 °C up to 50 °C; Walker and Stringer, 1987 and Junttila *et al*., 1988), although 45 °C is commonly accepted as the normal upper limit of growth. It can readily survive at extremely low temperatures and can withstand freezing (Johnson *et al.*, 1988).

While the organism shows its optimal growth at neutral pH, it can also grow over a wide pH range between 4.3 and 9.6 (Seeliger and Jones, 1986; Farber *et al*., 1989), but no growth was observed at pH 4 or below (Parish and Higgins, 1989).

Furthermore, *L. monocytogenes* has the ability to stand salt stress which is commonly employed as an agent for food preservation. Its high resistance to osmotic stress, including NaCl of up to 10 % (Walker *et al.*, 1990; Wemekamp-Kamphuis *et al.*, 2002) and its growth at high salt concentrations has mainly attributed to the accumulation of some organic compounds such as proline which it can use as compatible solutes (Wemekamp-Kamphuis *et al.*, 2002).

1.7.3 Biochemistry

After growth on agar plates, typical *Listeria* colonies are further identified using Gram stain, motility at 20–25 °C, catalase, oxidase, haemolysis and carbohydrate utilisation tests (Table 1.3) (Seeliger and Jones, 1986; Johansson, 1998; Becher *et al*., 2006).

1.7.3.1 API- System

Detection of *Listeria,* based on biochemical confirmation, is most commonly carried out using API Listeria system for identification that can be used to identify various *Listeria* strains at both species and subspecies levels. The API *Listeria* system contains dehydrated substrates, which allow enzymatic tests or sugar fermentations to take place, and eliminate the need for the CAMP test to confirm the haemolytic status of the organism (see below).

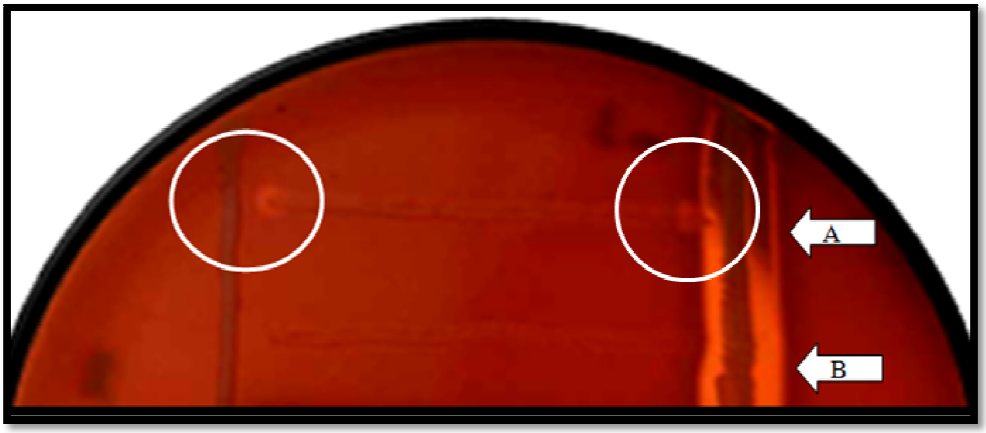
This innovative procedure has considerably reduced the time needed compared to conventional identification. The API Listeria test kit is easy to use, relatively rapid (McGowan, 1989) and inexpensive (Bille *et al*., 1992). It, therefore, appears to be a practically promising tool for the routine daily practices, especially those concerned with food and environment microbiology (Bille *et al*., 1992; Aznar and Elizaquível, 2008).

Although the organism does secrete a haemolysin, haemolytic activity observed when the cells are grown on blood agar is very weak and is described as β-haemolysis more characteristic of organisms that do not secrete haemolytic proteins (Mathakiya *et al.,* 2011). To overcome this problem of visualising the haemolytic reaction, the Christie–Atkins–Munch-Petersen

(CAMP) test has been the most important test for confirming the identity of member of the *Listeria* genus by enhancing the haemolytic activity of *L. monocytogenes* (Perrin *et al*., 2003*)*. This test is performed by streaking a β-haemolytic *Staphylococcus aureus* and a *Rhodococcus equi* in single lines parallel to each other on opposite sides of a sheep blood agar plate (Fig.1.1). The suspect *Listeria* isolates are then streaked at right angles, but not touching, to the streaks of the other organisms. After 12 - 18 h of incubation at 37 ℃, β-haemolysis produced by *L. monocytogenes* is seen to be enhanced near the point of the *S. aureus* streak (Mathakiya *et al*., 2011).

Although this is the classically accepted reaction, positive CAMP reactions between *L. monocytogenes* and *Rhodococcus equi* have been reported by Mckellar (1994) and FernandezGarayzaba *et al*. (1996) (Fig 1.1).

Figure 1.1: Illustration of CAMP test



L. monocytogenes is cross streaked on a blood agar plate between cultures of *Staphylococcus aureus* (right vertical) and *Rhodococcus equi* (left vertical). Taken from Aznar and Elizaquível, 2008. Upper line (A) shows *Listeria monocytogenes* (weak haemolysis is circled) and lower line (B) shows *Listeria innocua*.

Table 1.3: Biochemical identification and differential characteristics of *L. monocytogenes*

Test	Result	Comment
Gram stain	+	Short rod shaped cells, some longer filaments visible. Described as forming "Chinese letters"
Motility at 25 °C	+	Tumbling motility via peritricious flagellae
Motility at 37 °C	-	Very few or no flagellae not produced
Catalase	+	tests
Oxidase	-	
L-rhamnose	+	
Hydrolysis of Esculine	+	
CAMP (*R. equi*)	-	
CAMP (*S. aureus*)	+	

+ ***positive reaction or test result*** **—** ***negative reaction or test result***

Adapted from Seeliger and Jones, 1986; Liu et al., 2006; Mathakiya et al., 2011.

1.8 Isolation and Confirmation of *Listeria monocytogenes*

Many studies have been conducted to develop detection procedures for *L. monocytogenes*. Current conventional methods for the detection of *L. monocytogenes* rely mainly on selective enrichment media, followed by growth of the isolate on a selective medium, isolation of presumptive colonies and then biochemical and serological testing (Donnelly, 2002). So far no selective enrichment medium which is capable of differentiating between *L. monocytogenes* and other *Listeria* species is available. Generally the methods are labour-intensive and time-consuming, taking several days to complete.

Several international method standards are available for detection of *L. monocytogenes*, which involve one or two steps using typical enrichment broths and plating on selective agar media (e.g. International Standard Organisation (ISO), ISO 11290-1 (ISO, 1996) for detection enumeration, the International Dairy Federation (IDF) (Hitchins and Jinneman, 2011) and Nordic Committee on Food Analysis (NCFA, 1999).

ISO 11290 standards cultural methods are one of the most commonly used cultural methods for detection of *L. monocytogenes* include selective enrichment in broth such as Fraser broth, followed by the isolation of colonies on selective agar, (e.g. Oxford medium and PALCAM), biochemical characterization of suspect isolates colonies and serological confirmation steps.

In several studies, special enrichment and plating procedures for detection of *L. monocytogenes* prepared from different kinds of samples have been

compared with none of the selective media suggested being suitable for all purposes (Warburton *et al*., 1991; Waak *et al*., 1999).

Nowadays, the development of rapid detection and quantification methods for *L. monocytogenes* in various food products, as well as in different food processing and displaying environments, are urgently needed, both for food quality control assurance and for tracing listeriosis outbreaks within the food chain. Time required for the analysis and sensitivity are important limitations related to the usefulness of any microbial tests (Mandal *et al*., 2011). Significant efforts have been dedicated to the development of rapid detection methods, and different tests have been developed and suggested for this purpose.

Currently more molecular methods, such as antibody-based detection methods (ELISA) or molecular techniques (PCR or DNA hybridisation), have been applied to achieve quicker and more specific detection of *L. monocytogenes* (Manzano *et al*., 1996; Almeida and Almeida, 2000; Gasanov *et al*., 2005; Poltronieri *et al*., 2009) and have been developed to either support or replace traditional techniques (Deneer and Boychuk, 1991; Rossen *et al*., 1991; Niederhauser *et al*., 1992; Janzten *et al*., 2006). Many of these have been successfully used for the detection and identification of the presence of *L. monocytogenes* in various food products, and offer a shorter analysis time, lower detection limits, and higher specificity and potential for automation (Zottola 1994; Germini *et al*., 2009).

1.9 Detection Methods for *Listeria monocytogenes*

1.9.1 Rapid Methods

Rapid methods for monitoring various environments have been continuously developed. There are two main methods which have been developed to shorten the detection period required and to increase accuracy and specificity of detection of *L. monocytogenes* in food products: ELISA assays and PCR assays, which can currently be used to facilitate identification of *Listeria* spp.

1.9.1.1 ELISA assay

ELISA typically includes four principle steps, consisting of coating, blocking, reacting of antigen and antibody and detection. The format of these assays depends on the types of antibodies and antigens. The ELISA test is based on the use of a specific enzyme to detect the binding of an antigen (Ag) present on the cell surface to an antibody (Ab) (Voller *et al*., 1978) and the success of any ELISA result depends principally on the specificity of the antibody (Janzten *et al*., 2006). ELISA tests are the most common rapid methods used to detect pathogens in foods (Kim *et al*., 2005; Janzten *et al*., 2006).

The ELISA method is noted to have a high standard of specificity and sensitivity. Although ELISA offers an economical and suitable method for sensitive detection of low concentration compounds (Rittenburg, 1990), direct detection of pathogens in food is not possible and selective enrichment is required for at least 16-24 h in which detection of small numbers of organisms is required (Oladepo *et al*., 1992; Mandal *et al*., 2011). Specialized test kits to aid in the detection of pathogenic bacteria such as *Listeria,* are commercially

available. These are used as mass-screening methods to determine whether *L. monocytogenes* and other members of the species are present in either food or environmental samples following an enrichment procedure.

1.9.1.2 PCR assay

PCR (Polymerase Chain Reaction) tests can also be used for detecting *Listeria* in food samples, and are faster at providing identification results compared to the use of conventional tests. The first demonstration that PCR could be used to detect *L. monocytogenes* in food samples was by Wang *et al.* (1992), and since then it has been increasingly used for rapid identification of *Listeria* spp. and, more importantly, for the confirmation and differentiation of *L. monocytogenes* from other *Listeria* species (Gasanov *et al*., 2005).

There have been many studies conducted on *Listeria* detection using PCR techniques evaluated in milk, chicken, ground beef (Croci *et al*., 2004; Thomas *et al*., 1991), salmon and salmon products (Norton *et al*., 2001; Rodríguez-Lázaro *et al*., 2005), in cold salted (gravad) rainbow trout (Ericsson and Stålhandske, 1997), channel catfish (Wang and Hong, 1999), fish seafood products (Bansal *et al*., 1996; Gouws and Liedemann, 2005), smoked salmon (Simon *et al*., 1996; Becker *et al*., 2005), and environmental samples (Norton *et al*., 2001). Many of these studies focused on whether the detection event is affected when the sample used is in a specific food matrix compared to results using pure cultures. In many cases false-negative results were found when trying to detect low levels of *Listeria* directly from food samples because of the presence of PCR inhibitors (Bhaduri and Cottrell, 1998; Holko *et al*., 2002).

The reliability of PCR pathogen detection depends, in part, on the purity of the single DNA target molecule, since the activity of DNA polymerases is susceptible to inhibition by food components, selective enrichment media, or large amounts of non-target DNA, as shown for *Listeria* by Yang *et al*. (2007). Another limitation is that PCR assays cannot be conducted on DNA extracted directly from a food sample (e.g. 25 grams) due to the large amount of material and the low volume capacity of PCR reaction tubes and using large volumes in PCR reactions may decrease the efficiency of the PCR reaction. For instance Rodriguez-Lázaro *et al*. (2005) reported that at a concentration of 10^7 CFU g^{-1}, *L. monocytogenes* was not detected in raw or cold-smoked salmon samples and raw pork using a PCR method.

Some research studies have described sample treatments being performed prior to the PCR test in order to remove PCR inhibitors from a variety of food matrix, but generally PCR-based detection methods are carried out after selectively enriching samples to allow inhibitors to be removed by dilution and after the number of cells to be detected has increased (Gasanov *et al*., 2005).

The most frequently chosen target gene used in PCR detection tests for *Listeria* is 16S rRNA (Aznar and Alarco´n 2003; Somer and Kashi, 2003), due to the 16S rRNA gene being large enough for informatics purposes and also the function of the 16S rRNA gene over time has not changed (Janda and Abbott, 2007).

inlA - *inlB* (the operon encoding the virulence factors internalin A and internalin B; Aznar and Alarco´n, 2003; Chaturongakul *et al.,* 2008) and *hlyA*, encoding listeriolysin O (Churchill *et al.,* 2006).

Multiplex PCR, which is amplification of multiple targets in a single PCR experiment, allows the simultaneous detection of more than one target sequence in the same sample, such as *Listeria* and *Salmonella* (Li *et al*., 2000; Hsih *et al*., 2001) or *L. monocytogenes* and other *Listeria* species (Lawrence *et al*., 1994; Hudson *et al*., 2001; Wesley *et al*., 2002). This technique is most attractive for food analysis, to produce considerable savings in time, reagents and labour costs are all reduced (Gasanov *et al*., 2005). However, there are many problems associated with direct detection of *L. monocytogenes* in food, for example the PCR assays alone cannot provide live/dead differentiation (Flekna *et al.*, 2007).

1.9.2 Bacteriophage for Detection of Bacterial Pathogens

Bacteriophage, or phage, were discovered twice at the beginning of the 20th century, firstly by Frederick Twort and secondly by Félix d'Hérelle (Helvoort, 1992). In 1915, Twort incubated some colonies of bacteria (Gram-positive cocci) on agar plates and observed that after a long time the bacterial colony became transparent. He proposed several explanations but one of them was that an agent was causing the cells to lyse and that this might be viral in nature (Ackermann, 2003). Two years later (1917) d'Hérelle observed the action of bacteriophage when he was monitoring the lysis of *Shigella* cultures in broth,

and also he described local regions of bacterial cell lysis induced by viruses on agar plates (Ackermann, 2003). Now phage infecting over 140 bacterial or archaeal genera have been described and they are reported to be the most abundant life form on the plant (Maniloff and Ackermann, 1998; Ackermann, 2003, 2007;). As with all viruses, phage are infectious particles that undergo adsorption, to the host bacterial cell followed by injection of nucleic acid, replication and phage release. The highly specific interaction of phage with target bacterium is indicated by the formation of plaques only on certain host strains. The plaques are formed when the phage infects and destroys the host organism during its life cycle and the final plaque should not contain any viable bacteria (McNerney *et al*., 2004).

The International Committee on Taxonomy of Viruses has classified bacteriophage into families according to nucleic acid and morphology (http://www.ictvonline.org/). Phage consist of an outer protein capsid enclosing genetic material. This genetic material can be single-stranded DNA, double-stranded DNA, single stranded RNA or double stranded RNA (Guttman *et al*., 2005) and on the basis of this, bacteriophage have been classified into one order and 13 families. However phage morphology is still a key part of the classification process.

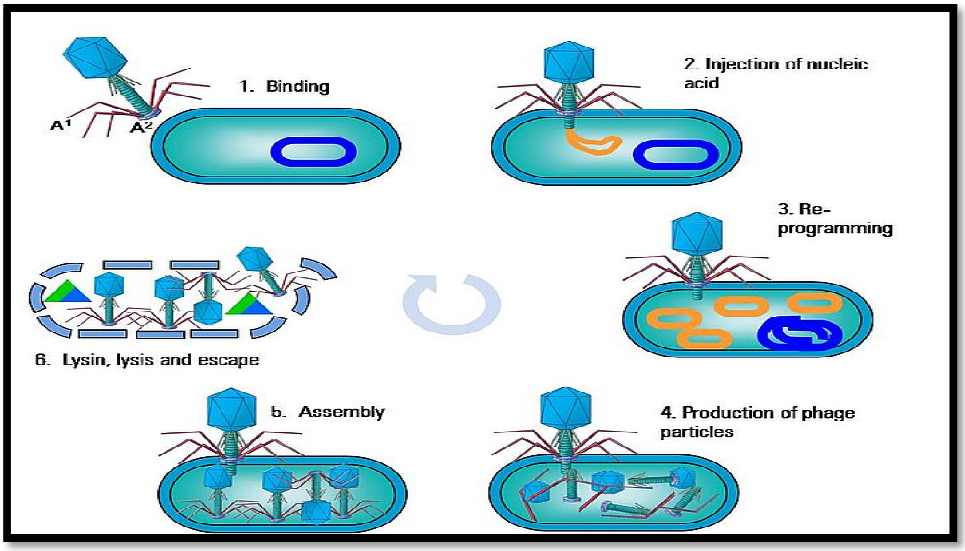
Since 1959 the electron microscope has been used for examining over 5100 phage. At least 96 % of phage that are isolated from the environment are tailed, and the tail structures are known to facilitate the binding of the virus to its host cell and delivery of the nucleic acid through the rigid cell wall. The tailed phage constitute the order *Caudovirales* and this comprises three families -

Myoviridae, Siphoviridae and Podoviridae - identified on the basis of similarities in virus morphology, replication, and assembly (McKinstry, *et al*., 2005). At present, there are six genera in the family Myoviridae (viruses with contractile tails), six in the family Siphoviridae (viruses with long, non-contractile tails) which is the predominant form isolated (61 % of tailed phages) and three in the family Podoviridae (viruses with short non-contractile tails).

1.9.2.1 Phage life cycle

Depending on their life cycles, phage can be one of two types, called lytic (virulent) or lysogenic (temperate). Lytic or virulent phage always begin to multiply inside the bacterial host after it infects the cell and this results in host cell lysis at the end of the replication cycle (Fig. 1.2). However when lysogenic or temperate phage infect cells, the nucleic acid is either integrated into the host bacterium's genome, or exists as an episomal element, resulting in a prophage within the cell and all its progeny (Gill and Abedon, 2003). The phage DNA is then replicated as part of the host genome for a period of time (the lysogenic cycle). The lysogenic cycle is one strategy for replicating the virus, but without destroying the host (Campbell, 1996; Strauch *et al*., 2007). In both cases the life cycle involves adsorption of the phage to, and recognition of, the bacterial cell wall after a random collision between the host and phage which is initially non-specific (Wang *et al*., 2000; Young *et al*., 2000; Molineux, 2006). The specific recognition and attachment with tailed phage starts when specialised adsorption structures, such as fibers or spikes, bind to specific surface molecules on their target bacterium.

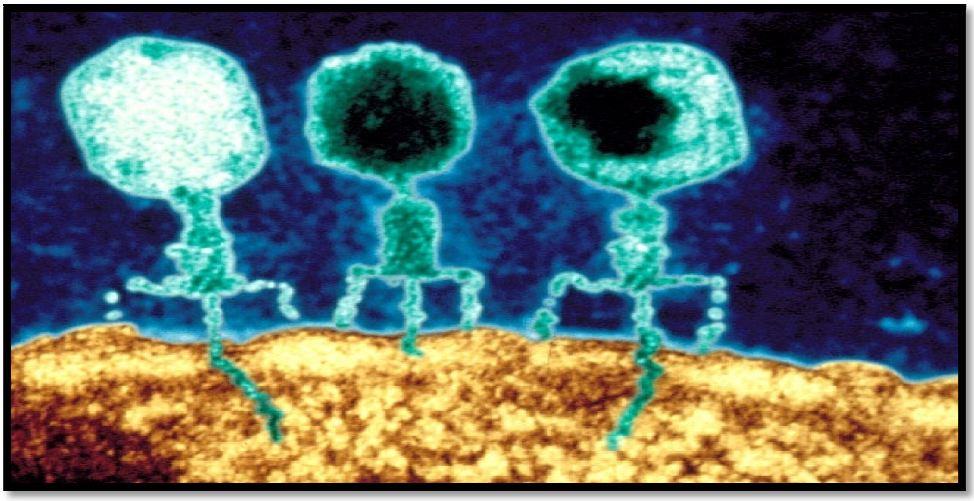
Figure 1.2: The Lytic Cycle: A phage infects the bacterium and lyses the cell and escapes



Reproduced from Hyglos GmbH:

(http://www.hyglos.de/en/technology/technologicalbackground/bacteriophage-biology.html)

Figure 1.3: The bacteriophage infects the bacterium to inject its genetic material into the cell



Reproduced from

(http://skiencestuff.blogspot.co.uk/2010/05/bacteria-have-immune-systems-role-of.html)

The outer membrane of Gram-negative bacteria differs in structure from the plasma membrane of both Gram-negative and Gram-positive micro-organisms (Rakhuba *et al*., 2010). In Gram-negative bacteria any surface protein, lipopolysaccharide or oligosaccharides can serve as bacteriophage receptor, whilst in Gram-positive the receptors are most commonly muramic acid residues (Lindbergh, 1973; Dmitriev *et al*., 1999; Holtje, 1998; Rakhuba *et al*., 2010). After binding between the phage and a specific receptor on the host cell, phage inject their nucleic acid through the host cell wall into the cell, leaving the protein capsid outside (Fig. 1.3). During the penetration process, small holes are produced in the bacterial cell wall and if many phage absorb to a host cell, the cell wall may be sufficiently weakened and causes lysis of the cell. This phenomenon is called "lysis from without".

When the phage DNA enters the cell, the phage nucleic acid is converted into a circular DNA form by a variety of mechanisms, including the annealing of sticky ends (e.g. phage *Lambda*), through recombination of terminally redundant sequences (e.g. phage T4) or by linear end protection (e.g. phage PRD1).

Once the phage begins to replicate in the host cell the biosynthesis of phage components takes place. During the replicative phase, host cell protein and DNA synthesis is often inhibited; the phage DNA takes over the protein synthesis of the bacterium. The proteins are formed into the structural components of the new phage particles – i.e. the heads and the tails are assembled in the host cytoplasm. Nucleic acid is then packaged inside the head and then tail is added to the head. During this process the head expands

and becomes more stable and the internal volume of the packaged DNA increases. Finally, during the assembly, or maturation, process the head and tails are joined together after DNA encapsidation (Ackermann and DuBow, 1987; Maloy *et al*., 1994; Kutter *et al*., 2005) and this can happen spontaneously or with the help of specific phage-encoded enzymes.

As the final step after assembly, the host cell is lysed and releases the phage progeny. Phage are reproduced very quickly, and on host lysis the number of particles released per infected bacterium may be in the hundreds (Hayes, 1968). The number of new phage produced depends on the species and conditions; however, each "parent" phage may produce on average 50 - 200 "daughter" phage per lytic cycle (Carlton, 1999).

1.9.3 *Listeria*-specific phage

Phage are extremely host-specific but are known to possess different host ranges; they can infect either a group of related bacteria, specific species within a genus or specific strains within a species. About 400 bacteriophage specific for *Listeria* (listeriaphage) have been isolated from foods, silage, and lysogenic strains or environmental sources such as sewage (McKinstry, *et al*., 2005; Kim *et al*., 2008). They are characterised as having dsDNA genomes, ranging from 36 to 135 kbp in size with a G+C content of 34.7 - 40.8 mol % (Rocourt, 1986; Loessner *et al*., 1994; Loessner *et al.*, 2000; Carlton *et al*., 2005). Although, many of these bacteriophage are specific for *L. monocytogenes*, in addition they are able to infect other members of the genus, such as *Listeria innocua,*

Listeria ivanovii, *Listeria seeligeri* and *Listeria welshimeri* (Hagens and Loessner, 2007).

Many *Listeria* phage have been investigated by electron microscopy and the structure of all virions studied to date are tailed phage. The majority of these phage are temperate, with two notable exceptions being A511 (Zink and Loessner, 1992) and P100 (Carlton *et al*., 2005). A511 is a Myovirus with a non-flexible, contractile tail that is 200 nm long with a contractile sheath around the inner tail tube and an isometric capsid of 88 nm in diameter (Loessner and Scherer, 1995; McKinstry, *et al.,* 2005). It has a relatively large genome (genome size, approximately 116 kbp; Loessner *et al*., 1994) and a very broad host range and it can lyse about 95 % of *L. monocytogenes* strains of serovars 1/2a and 4b (Loessner *et al*., 1996; 1997). It has a relatively large genome (Loessner *et al.,* 1996); P100 is similar to the characterised phage A511 (Carlton *et al*., 2005) but has a genome size of 131 kbp (Coffey *et al*., 2010).

Several studies have suggested that the virulent *Listeria* phage could be used as biological control agents for *Listeria* during food processing and storage (Leverentz, 2003 and 2004; Hudson *et al*., 2005), and commercial products have been introduced into the market (Garcia *et al*., 2008). In 2006 the FDA (U.S. Food & Drug Administration) approved the application of a listeriaphage mixture for use on the surface of meat and poultry products and ready-to-eat foods (http://www.foodnavigator-usa.com/Business/FDA-approves-viruses-as-food-additive-for-meats), and also Europe and New Zealand approved the use of Listex P100 solution to reduce *L. monocytogenes* surface contamination on

raw fish (http://www.foodproductiondaily.com/Quality-Safety/Dutch-firm-sets-sights-on-worldwide-approval-after-EFSA-backs-Listeria-combatting-solution).

1.9.4 Phage detection assays

The rapid detection of pathogens in food and environment is critical for ensuring the safety of consumers. Many of these methods are expanded to isolate, concentrate, and purify the bacteria from the sample matrix before detection. Recent challenges in technology make detection and identification faster, more specific and more sensitive than conventional assays. These new methods are often referred to as rapid methods. Rapid and accurate detection methods based on the interactions of phage and their host bacteria have been developed to indicate the presence of low counts of bacterial cell numbers within a few hours (De Siqueira *et al*., 2003).

Cherry *et al*. (1954) first developed a method for the identification of *Salmonella enterica* by means of a specific bacteriophage called Felix-01. Later on several methods have been developed by using the bacteriophage for detection of *Salmonella* based on the interactions of phage Felix-O1 (Hirsh and Martin, 1983; Sillankorva *et al*., 2010). Another use of phage to detect cells was described by Bennett *et al*. (1997), who used a biosorbant that consisted of *Salmonella*-specific phage immobilised to a solid phase for the concentration

of *Salmonella* from food materials and they suggested reduction of the time of analysis (Schmelcher and Loessner, 2008).

Phage amplification technology (Fig. 1.4) is one of the alternative techniques that has proved successful due to its rapidity and sensitivity and is also considered to be a quantitative and cost-effective method that works well with foods (Rees and Dodd, 2006; Stanley *et al*., 2007; Botsaris *et al*., 2010). Phage amplification assay is based on the specific interaction of a phage and its host bacterial cell and was developed and first described about 20 years ago (Stewart *et al*., 1992; Stewart *et al*., 1998; Park *et al*., 2003). The method is well established as a method to detect slow growing pathogenic *Mycobacterium* by exposing a sample suspected to contain target cells to a host-specific phage (Jassim and Mazen, 2007). This assay has been produced as a commercial kit for the detection of *Mycobacterium tuberculosis* in human sputum samples (www.biotec.com; Rees and Botsaris, 2012)

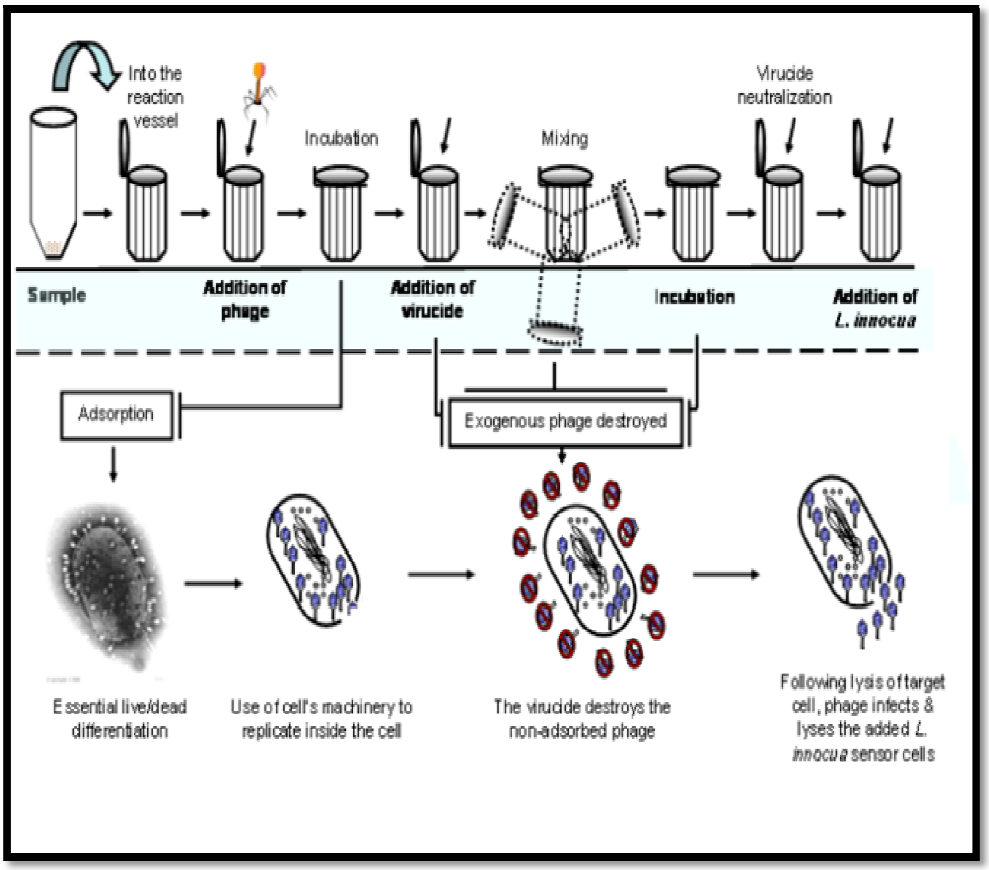
To perform the assay the sample is incubated for the time necessary for the cell to be infected by the bacteriophage and then the mixture is treated to destroy any viruses that have not infected the host cell. This may be a physical, enzymatic or chemical treatment (Marei *et al*., 2003). The infected bacteria are then mixed with a culture of rapidly growing Mycobacterial cells which the phage can also infect, and amplification of the phage occurs during the lytic replication cycle (Park *et al*., 2003).

One of the first reports of the growth of bacteriophage was by Ellis and Delbrück (1939) who concluded that the replication of phage in a lawn of

bacterial cells results in the formation of plaques, which take the form of a clear zone that results from the lysis of the bacteria in the lawn. Each plaque arises from a single infectious phage (Stanley *et al.*, 2007). In the phage amplification assay the bacteriophage undergoes rapid cycles of infection, replication and lysis using the host cells in the lawn, resulting in the formation of plaques (Figure 1.4). Hence each plaque present represents one target bacterium in the original sample (Marei *et al.*, 2003; Schmelcher and Loessner, 2008).

A key component of this assay is a potent virucidal agent, which is an important step for successful application of this method. Several virucidal agents derived from natural plant extracts, such as pomegranate rind extract (PRE) *Viburum plicatum* or loose-leaf tea extract have been described. When used in combination with 4.3 mM ferrous sulphate PRE was shown to inactivate *Pseudomonas aeruginosa* bacteriophage and reduced phage stocks by $\log_{10}$ (Stewart *et al.*, 1995; Stewart *et al.*, 1998). Moreover date pit extracts were described by Jassim and Mazen (2007), to have a strong ability to inhibit the infectivity of *Pseudomonas* phage ATCC 14209-B1. However the virucidal compound commonly used in this assay is ferrous ammonium sulphate (McNerney *et al.*, 1998; Park *et al.*, 2003). Furthermore the use of essential oils and plant extracts has also been reported (Rees and Voorhees, 2005), whereas De Siqueira *et al.* (2006) described using various tea infusions for phage inactivation.

Figure 1.4 Overview of Phage Amplification Assay



Taken from El Emam, 2010

The phage amplification assay comprises: phage infection of target bacterium; destruction of exogenous phage; amplification of phage within infected host and plaque formation from infected host with the aid of helper bacteria.

Stanley et *al.* (2007) have further developed this method by combining this test with PCR amplification (Phage-PCR) in order to determine the genetic identity of any cell infected by the phage. The advantages of the combined phage-PCR assay over other PCR-based detection methods is the cell must be viable for the phage to replicate, and therefore provides live/dead differentiation which standard PCR assays do not achieve, and the simplicity of preparation of the sample, since it uses the phage selectively to lyse only the target cell and the DNA from the single host cell is held in a fixed place so it is easy to isolate.

1.10 Research Objectives

Based on the success of the phage detection assay for the detection of *Mycobacterium* in human sputum samples and the recent modification that allows the molecular identification of the cell detected, the present study was designed to develop a phage-based detection method for *L. monocytogenes* in foods.

Specifically the aim was to develop a new phage-based assay procedure that would considerably shorten the long analyses time required by the conventional standard procedure ISO 11290-1 for *Listeria* isolation and identification. The design of the method would be to allow detection of low numbers of cells, with an intention to reduce both time and laboriousness of the work. To do this it was planned to use PCR amplification of signature sequences. Based on the above mentioned considerations, five objectives were formulated:

I. To concentrate and propagate two bacteriophage (23074-B1 and A511) and also to evaluate different virucidal agents for use in the phage detection assay.

II. To optimise the molecular multiplex-PCR-based method in order to detect and identify all the species of the genus *Listeria* simultaneously from plaque samples within a short period.

III. To combine the plaque PCR technique with the phage-based detection assay in order to allow the rapid and specific detection of viable *L. monocytogenes.*

IV. To examine the performance of the efficacy of the new assay by using Stilton cheese samples that are a British internally semisoft blue cheese obtained from pasteurised cows' milk. These products often provide excellent conditions for growth.

V. To isolate and identify *L. monocytogenes* by combining the plaque PCR technique with the phage-based detection assay to allow rapid detection of viable *L. monocytogenes* in different kinds of foods.

CHAPTER 2

MATERIALS AND METHODS

2.1 Solutions and Media

All the solutions and media were prepared using reverse osmotic (RO) water, which then was sterilised by autoclaving at 15 p.s.i (121 °C) for 20 min.

2.1.1 Lambda Buffer

This buffer consisted of 6 mM Tris-HCl (pH 7.6), 10 mM $MgSO_4$ and 0.05 % (w/v) gelatin in RO water and autoclaved.

2.1.2 Maximum Recovery Diluent (MRD)

The dehydrated MRD (Oxoid CM733) was suspended in RO water at 9.5 g l^{-1} and then autoclaved.

2.1.3 Virusol

Virusol was prepared according to the manufactur's instructions (Biotec Laboratories Ltd, Ipswich, U.K). One vial of *FASTplaque*TB^{TM} virusol was dissolved in 5 ml of sterile distilled water. The final solution was 10 mM Ferrous Ammonium Sulphate.

2.1.4 Acetic Acid (CH_3COOH)

CH_3COOH was prepared by adding sterile distilled water to CH_3COOH to a final concentration of 0.31 % or 0.62% or 0.93 % (v/v), as required.

2.1.5 Ferrous Sulphate ($FeSO_4$)

To prepare 4.3 mM ferrous sulphate, a stock solution of 27.8 $FeSO_4$ g $l^{-1}$was prepared, and 4.3 ml from stock solution was added to 95.7 ml of sterile distilled water and sterilised by filtration, a membrane with a porosity of 0.2 µm (Stewart *et al*., 1998)

2.1.6 Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide was diluted in sterile distilled water to 3.5 % (v/v) and then used.

2.1.7 Different Type of Teas

Eight types of loose-leaf tea (Table 2.1) were used to prepare infusions (7 % w/v) by additing sufficient RO water to the tea solids and boiling for 10 min. The infusion was then filtered (Whatman Grade No. 2 Filter Paper, Whitman International Ltd.), autoclaved and stored at 4 °C. The tea infusions were also tested at different concentrations using the same method used above.

Table 2.1: Eight types of loose-leaf tea used in this study

Sample No.	Tea Type	Details as described/ weight	Purchased From
1	Green tea, Gunpowder Leaf Tea	Smooth, classic 125g	Whittard of Chelsea Bridlesmith Gate, Nottingham
2	Green tea, Sencha Leaf Tea	Famous Japanese Leaf Tea. 125g	»
3	ASSAM, Leaf Tea	Strong, firm and rich malty Indian tea. 125g	»
4	Ceylon, Leaf Tea	Beautifully smooth, fresh golden tea, 125g	»
5	Kenya, Leaf Tea	Strong, firm and rich malty-black tea, 125g	»
6	DARJEELING, Leaf Tea	The (champagne) of teas, aromatic, 125g	»
7	Earl Grey, Leaf Tea	Light, aromatic blend scented, 125g	»
8	Organic Green Loose Leaf Tea	50 g	ASDA Supermarkets
9	Thyme	17 g	ASDA Supermarkets

2.1.8 Brain Heart Infusion Broth (BHI) and BHI AGAR

BHI broth powder (Oxoid) was suspended in RO water at 37 g l^{-1} and was autoclaved; or was supplemented with agar (Oxoid) to 1.5 % (w/v) to produce agar plates.

2.1.9 Tryptose Soya Broth (TSB) and TSA Agar

TSB powder (Oxoid) was suspended in RO water at 30 g l^{-1} and autoclaved; or was supplemented with agar (Oxoid) to 1.5 % (w/v) to produce agar plates.

2.1.10 Fraser Broth

Fraser broth (CM 895 Oxoid) was prepared by adding 1 litre of RO water to 57.4 g of dehydrated medium and this was then sterilised by autoclaving. One vial of Fraser supplement was then reconstituted by adding 5 ml of sterile RO water and ethanol in a 1:1 ratio and aseptically (Flame sterilization) adding it to the cooled, autoclaved Fraser broth.

2.1.11 Half Fraser Broth

Half Fraser supplement (CM 895, supplement SR 166E Oxoid) was made by adding 12.9 g of medium into 225 ml of RO water and autoclaving. Sterile RO water (4 ml) and ethanol in a 1:1 ratio was then added to Half Fraser supplement, and all of this was finally mixed in with the sterilized Half Fraser broth.

2.1.12 Palcam Agar

PALCAM agar (CM 877 Oxoid) was made by dissolving 69 g of the dehydrated medium in 1 litre of RO water and then autoclaved. The supplement for PALCAM (SR 150E) was rehydrated by adding 2 ml of sterile RO water to the vial, before adding the supplement aseptically to the sterile PALCAM agar. Finally, 20 ml aliquots of the agar were aseptically poured into Petri dishes.

2.1.13 Listeria Selective Agar (Oxford)

Listeria selective agar (CM 856 Oxoid) was also prepared by dissolving 55. 5 g of the dehydrated medium into 1 litre of RO water and then autoclaved. A

Listeria selective agar supplement (SR 140E) was then rehydrated with 5 ml of sterile RO water and ethanol (70 %) and was aseptically added to the sterile Listeria selective agar. Similarly, 20 ml aliquots of the agar were then aseptically poured into Petri dishes.

2.1.14 Preparation of D10 Medium

The defined medium D10 was prepared as described by Trivett and Meyer (1971). In order to prevent formation of precipitates, sterilised (microfiltration) vitamins were used in the medium solution. The prepared medium was assembled as follows:

a. 8.50 g of K_2HPO_4 (Fisher Scientific), 1.5 g of NaH_2PO_4. H_2O (BDH) and 0.50 g of NH_4CI (Sigma) were dissolved in 500 ml of RO water ;
b. 0.2 of NaOH (Fisher Scientific) and 0.48 g of nitrilotriacetic acid were dissolved in 40 ml of RO water;
c. 0.048 g of $FeCl_3.6H_2O$ was dissolved in 40 ml of RO water ;
d. The $FeCl_3.6H_2O$ solution was mixed with the sodium nitrilotriacetic solution;
e. 0.41 g of $MgSO_4$. H_2O (Fisher Scientific) was dissolved in 30 ml of RO water ;
f. The produced solutions from steps d and e, the amino acids (Acros Organics) (Appendix 1), and 390 ml of reverse osmosis water were added to the solution produced from step a to make a volume of 1000 ml and was then autoclaved. Vitamin solution (10 ml of 100× stock) (described below) was added after this had cooled down to room

temperature. The pH of the medium was neutral (e.g. between 7.2 and 7.5).

The vitamin solution (100×) was prepared as follows. α-lipoic acid (5 mg; Fluka Biochemika) was dissolved in 200 ml of 70 % ethanol. Biotin (5 mg; Fisher Scientific), thiamine (50 mg; Sigma) dissolved in 125 ml of 95 % ethanol were mixed with 2 ml of the α-lipoic acid/ethanol solution, and the volume adjusted to 500 ml and the solution was filtered sterilised using a 0.45 µl membrane filter (Millipore).

The D10 medium was supplemented with 10 ml of a 20 % (w/v) glucose solution sterilized by autoclaving. The glucose was used to replace fluid lost during autoclaving of the base solution and therefore no adjustment for volume was made. Contents of D10 media are listed in Appendix 1.

2.1.15 Preparation of MCDB Medium

The MCDB 202 medium was prepared as described by Chavant *et al*. (2002) by dissolving 9.9 g of MCDB 202 medium (US Biological) in 900 ml RO water and stirring gently until completely solubilised. RO water was added to bring the solution to 1 litre after which it was supplemented with 1 % (w/v) yeast nitrogen base without amino acids (Difco) and 3.6 g l^{-1} glucose (Fisher Scientific). The medium was then sterilised using 0.2 µm membrane filter. Contents of MCDB 202 medium are listed in Appendix 1.

2.1.16 NAO Antibiotic Supplement

NAO antimicrobial supplement (Aztreonam 30 µg ml^{-1}, Oxacillin 2 µg ml^{-1} and Nystatin 50 IU ml^{-1} final concentration; Biotec laboratories) was prepared and used according to the protocols provided by the manufacturer.

2.1.17 CF Antibiotic Supplement

To prepare the Cycloheximide and Fosfomycin (CF) antibiotic supplement a solution of 40 mg ml^{-1} Cycloheximide, 1 mg ml^{-1} Fosfomycin solution in 70 % ethanol was prepared. This was stored in 1 ml aliquots at -20 ºC until used. 1 ml of CF solution was added to 100 ml of BHI agar (Section 2.1.8).

2.1.18 Top Layer Agar

BHI broth powder (Oxoid) was suspended in RO water at 37 g l^{-1}, and then the prepared solution was supplemented with 4.0 or 0.5 or 6.0 or 7.0 g l^{-1} agar (Oxoid) as required, dissolved and dispensed in 5 ml volumes into bijous tubes and autoclaved.

2.2 Bacterial and Bacteriophage Strains used

Table 2.2 Bacteriophage strains

Strain	Source
ATCC 23074-B1	ATCC*
A511	Prof. M. Loessner, ETH Zurich, Swizterland

**ATCC: American Type Culture Collection*

Table 2.3 Bacterial strains used

Strain	Serotype	Source
L. monocytogenes WSLC 1042	4b	Weihenstephan *Listeria* Collection
L. monocytogenes EGD-e	1/2a	Clinical isolate
L. monocytogenes ATCC 23074	4b	ATCC* Clinical
L. monocytogenes 1	1/2 a	Laboratory Collection; Milk
L. monocytogenes 3	1/2c	Laboratory Collection; Milk
L. monocytogenes 10	1/2a	Laboratory Collection; Food
L. monocytogenes 13	4b	Laboratory Collection; Food
L. monocytogenes 27	1/2a	Laboratory Collection; Food
L. monocytogenes 10403S	1/2a	Clinical isolate
L. innocua NCTC 11994	-	NCTC**
L. ivanovii NCTC 11846	-	NCTC**
L. seeligari NCTC 11856	-	NCTC**

**ATCC: American Type Culture Collection*
***NCTC: National Collection of Type Cultures*

2.3 Cultivation of Bacterial Strains and Propagation of Bacteriophage

2.3.1 Storage of Bacterial Strains

To create working cultures, all *L. monocytogenes*, *L. innocua, L. ivanovii* and *L. seeligari* were streaked onto BHI agar plates from a single colony and stored at 5 ºC for future use.

2.3.2 Preparation of Bacterial Cultures

Liquid cultures of *L. monocytogenes* and *L. innocua* were prepared by inoculating a single colony into 20 ml BHI or TSB in a sterilised Pyrex conical flask and then incubating at 37 ºC with continuous shaking (200 rpm) overnight.

2.3.3 Preparation of Cultures Using Minimal Growth Media.

Bacterial cultures were prepared by inoculating a single colony into 20 ml BHI and incubated at 37 ºC with continuous shaking (200 rpm) overnight. One ml of these cultures was added to 20 ml of D10 (Section 2.1.14) or MCDB 202 (Section 2.1.15) media in a sterilised Pyrex conical flask and then again incubated at 37 ºC with continuous shaking (200 rpm) overnight. Cells were concentrated by centrifuging (885 ×*g*, 10 min, room temperature) and then resuspended in 5 ml of the same growth medium

2.3.4 Preparation of Bacterial Lawns

After cooling 5 ml aliquot of soft top agar (prepared as in Section 2.1.18) to around 45 ºC, the solution was mixed with 100 µl (approx. 1×10^9 cfu ml^{-1}) of

L. monocytogenes culture (Section 2.3.2). The agar and bacteria was then dispensed onto BHI agar plates (Section 2.1.8) and was allowed to solidify at room temperature.

2.3.5 Phage (23074-B1 and A511) Titration

Bacteriophage were enumerated by using Miles and Misra technique (Atterbury *et al*., 2003). The bacteriophage suspension was serial diluted in ten-fold steps using lambda buffer (2.1.1). A lawn of host strains was prepared (Section 2.3.4) and triplicate drops of each phage dilution (10 µl) were pipetted onto the surface of the agar. The plates were then incubated at 40 min at room temperature to allow excess liquid to be absorbed and afterwards the plates were further incubated at 30 °C for 18 – 24 h. The number of plaques were counted and calculated, and the phage titre expressed as pfu ml^{-1}.

2.3.6 Producing of Phage in Liquid Culture

A portion (20 ml) of overnight liquid culture of *L. monocytogenes* WSLC 1042 (Section 2.3.2) was diluted 1 in 50 to give an optical density (OD_{600nm}) of ≈ 0.05 (~1×10^7 cfu ml^{-1}). Then phage (23074-B1 or A511) were added to achieve the required multiplicity of infection (m.o.i) and the culture incubated at 37 °C in an orbital shaker. Growth was measured every 45 min by determining the optical density at 600 nm using spectrophotometer (CEIL CE 2021) until lysis occurred (~ 5 h). The resultant phage lysate was stored at 5 °C overnight. The number of bacteriophage in the lysates was enumerated as described in Section 2.3.5.

2.3.7 Precipitation of Phage Lysate Using Polyethylene Glycol (PEG)

The phage lysate prepared as described in Section (2.3.6) was then centrifuged (5000×*g*, 15 min, 5 °C) to remove cell debris and any unlysed cells. Polyethylene Glycol (PEG 8000) was added to the phage suspension at a final concentration of 10 % (w/v) and dissolved gently at room temperature. This was stored over night at 5 °C, and centrifuged (11,000 ×*g,* 10 min, 4 °C) to concentrate the phage. Phage pellets were resuspended in the appropriate quantity (1/40th of the original phage lysate volume) of Lambda buffer (Section 2.1.1) and stored at 5 °C. The titre of phage was determined as described in Section 2.3.5

2.4 Treatment of cells or phage with Virucide

For treatment of cells, *L. monocytogenes* WSLC 1042 cultures (1 ml) grown overnight in BHI broth (Section 2.3.2) were centrifuged in a microfuge at 10960 x*g* for 5 min. The cell pellet was resuspended in 0.1 ml Lambda buffer. Aliquots (100 µl) of suspension were then transferred into sterile Eppendorf tubes. Cells were treated with 100 µl of virucide solution (see appropriate sections for details) and incubated for 20 min at room temperature. For treatment of the phage by the virucides, samples of the phage (100 µl) were added to 0.1 ml of MRD or Lambda buffer in sterile Eppendorf tubes and were then treated with 100 µl aliquots of virucide (see appropriate sections for details) and incubated for 20 min at room temperature. The titre of the phage

suspension was determined before and after adding the virucide as described earlier in Section 2.3.5.

2.5 *Listeria monocytogenes* Phage Amplification Assays

To prepare standard inocula for the phage assay *L. monocytogenes* WSLC 1042 culture (100 µl; Section 2.3.2) was mixed with 900 µl Lambda buffer (Section 2.1.1) and ten-fold dilutions prepared using Lambda buffer. Aliquots (500 µl) of each dilution were transferred into a sterile reaction vessel. A511 phage (100 µl; 10^9 pfu ml^{-1}) was added to the prepared samples and these were mixed in a rotating shaker (Grant) at 60 rpm for 3 min. These samples were then incubated statically at 37 ºC for 1 h to allow infection to proceed. Aliquots of virucide (500 µl) were added and the samples were again mixed (60 rpm, 3 min) and were then incubated at 20 °C for 20 min). To this, 100 µl portions of O/N *Listeria innocua* helper and 5 ml of BHI soft agar (Section 2.1.18) was then added and poured onto BHI agar (Section 2.1.8) and were incubated at 30 °C for 18 – 24 h and then the number of plaques produced was recorded. As a negative control 500 µl of Lambda buffer containing no *L. monocytogenes* cells was used.

2.6 Isolation of *Listeria monocytogenes* in Cheese

The detection of *L. monocytogenes* was performed according to ISO 11290-1-1996 method (ISO, 1996). 0.1 ml containing 20 ± 5 cfu were added into 25 g of Stilton cheese (Supermarkets, UK), and mixed with 225 ml of half Fraser

broth (Section 2.1.11), and homogenised by a laboratory Stomacher (Stomacher 400, England) at 230 rpm, for 2 min. The samples were incubated at 30 ºC for 22 h. Aliquots of 80 ml of samples were centrifuged (885 ×g, 10 min, room temperature) were resuspended in 20 ml of Fraser broth (Section 2.1.10), and then were incubated at 37 ºC with continuous shaking. Samples (1 ml) were then removed after 2, 4, and 7 h for analysis, centrifuged at 10960 ×*g* for 5 min, and resuspended in 0.1 ml Lambda buffer. These samples were then used in the phage detection assay. Parallel samples, aliquots of 0.1 ml of these samples were spread onto the surface of the PALCAM agar or Listeria selective agar for 18-24 h at 37 ^{o}C.

Characteristic black or dark green colonies surrounded by a black "halo" were considered to be presumptive *L. monocytogenes* colonies and their identity confirmed by carrying out further tests (e.g. Gram staining, Catalase, Oxidase, β-haemolysis and Hydrolysis of Esculin) (Sections, 2.8-2.9).

2.7 Conventional Plating Procedures For Detection of *Listeria* spp.

Standard plating procedures were conducted as reference methods in this study for validation of the assays developed. These experiments were carried out according to the procedures described in the ISO- 11290-1 (ISO 11290-1 ISO, 1996). In general, samples of foods (25 g of minced meat or pasteurised milk or turkey meat or smoked salmon or camembert cheese) were homogenised in a stomacher with 225 ml of half-Fraser broth (CM 895, supplement SR 166

Oxoid) for 2 min. Samples were inoculated with *L. monocytogenes* (to give approx. 1 cfu ml^{-1}; added 20 cfu (± 5) of *Listeria* to 25 g of sample). Cells were then concentrated 4-fold by centrifugation at 885 ×g for 10 min in Fraser broth. Samples (1 ml) were then removed after 2, 4, and 7 h for analysis, centrifuged at 10960 ×*g* for 5 min, and resuspended in 0.1 ml Lambda buffer. These samples were then used in the phage detection assay. Parallel samples, aliquots of 0.1 ml of these samples were spread onto the surface of the PALCAM agar or Listeria selective agar for 18-24 h at 37 ^{o}C.

2.8 Gram Stain

A smear of overnight cultures of the test isolate (Section 2.3.1 and 2.3.2) , was heat fixed on a glass microscope slide by passing the slide over a Bunsen flame 15-20 times. Afterwards, the prepared slides were placed on a rack and placed into crystal violet solution (2 g of crystal violet dissolved in 20 ml of 95 % ethanol. This solution added to 80 ml of a 1% Ammonium Oxalate solution) for 1 min. The slides were then washed with water for 5 s, and then were placed in the iodine solution (1 g iodine and 3 g potassium iodide in 300 ml distilled water) for 30 s. The slides were then washed again with water for 5 s before placing in methanol for 1 min.The slides were then stained using the safranin reagent (2.5 g safranin in 10 ml 95 % ethanol) for 30 sec and washed with water. Finally, the slides were blotted dry and examined under the light microscope using a 100× oil immersion lens.

2.9 Biochemical Identification Tests

2.9.1 Haemolysis Test

Organisms for testing were recovered from the PALCAM and Oxford agar, and then were streaked for single colonies onto the blood agar plates. The plates were then incubated for 24 h at 37 ^{o}C under either microaerophilic conditions, for organisms isolated from PALCAM, or aerobic conditions for Oxford agar. Two types of haemolysis were recorded: Alpha haemolysis which forms a narrow greenish zone of clearing around colonies, and beta haemolysis which forms a zone of complete clearing round colonies.

2.9.2 Catalase Test

A catalase test was performed to differentiate *L. monocytogenes* from other *Listeria* species by picking a colony from the surface of a Listeria selective agar or PALCAM agar plate using a sterile loop and transferring the cells onto a clean microscope slide and a drop of hydrogen peroxide solution (3 % v/v) was added onto the cell sample and the appearance of bubbles noted within 30 s of addition of the H_2O_2 solution.

2.9.3 Motility Test

Using a straight wire loop, suspect colonies were stabbed into the centre of the tube containing motility test medium to a depth around 5 mm and incubated at 25 °C for 18-24 h (ISO 11290-1:1 1996). The appearance of umbrella shaped growth zones on the motility test medium near the microaerophilic subsurface of the medium were recorded as positive for motility.

2.9.4 Oxidase test

The oxidase reagent was used according to the manufactur's instructions (Microbact oxidase strips, catalogue no. MB0266A). One strip containing N, N-dimethyl-p-phenylenediamine oxalate was touched directly onto the colonies on the plate. A positive reaction was recorded when a blue or purple colour appeared within 30 s at 22 °C.

2.10 Molecular Identification Methods

2.10.1 Preparation of Bacterial Chromosomal DNA Using Lysis

A colony of fresh overnight bacterial culture was added to 10 µl of sterile RO water in a sterile microfuge tube and was heated at 95 °C for 5 min in a heating block (Stuart Scientific). Samples were then centrifuged (13000 ×*g,* 5 min room temperature) in a microcentrifuge (Biofuge Pico; Heraeus, Pagnell, UK), and the resultant supernatant was extracted and frozen to -20 °C to be used as the template DNA for PCR amplification.

2.10.2 Preparation of Plaque DNA

Plaque DNA was excised (~10 µl) from the agar by using a sterile Gilson (1000 µl) micropipette tip. The agar plaque was transferred into a sterile Eppendorf tube, mixed with 10 µl of sterile distilled water and heated at 95 °C for 5 min (Modified from Stanley, 2007). The sample was frozen to -20 °C to freeze the agar and the samples were then thawed. DNA to be used as the template for PCR amplification was recovered from the supernatant after centrifuging (13000 ×*g,* 5 min room temperature).

2.10.3 Preparation of Genomic DNA

A GenElute™ Bacterial Genomic DNA Kit (Sigma) was used to isolate pure genomic DNA. Effective lysis was achieved by re-suspending plaque DNA in 10 µl (one plaque with 10 µl SDW), heating at 95 °C for 5 min and centrifuged at 13000 x*g* for 2 min.

20 µl RNase A (20 mg ml^{-1}; Sigma) was added and incubated at room temperature for 2 min, followed by the addition of 20 µl proteinase K.

GenElute™ columns (Sigma) were rinsed and centrifuged at 12,000 × *g* for 1 minute.

Absolute ethanol (200 µl) was added into the cell lysates and the solutions were mixed.

Lysates were loaded onto columns and eluted by centrifuging at 6500 ×*g* for 1 min (room temperature). Columns with bound DNA were washed twice with 500 µl wash solution (W0263, Sigma). Bound DNA was subsequently eluted with in 200 µl Elution buffer (10 mM Tris-HCl, and 0.5 mM EDTA [pH 9.0];

Sigma) by centrifugation at 6500 ×*g* for 1 min (room temperature). 10 µl of purified DNA was examined using gel electrophoresis on 0.8 % (w/v) agarose gel (Section 2.10.5) at 70 V for 30 min.

2.10.4 DNA Amplification by Multiplex-Polymerase Chain Reaction (mPCR)

The molecular identification of *L. monocytogenes* was carried out using the method described by Somer and Kashi (2003). The template DNA (chromosomal DNA or plaque DNA) was extracted from the colonies or plaques using methods described in Sections 2.10.2 - 2.10.3. A 25 µl multiplex PCR reaction was prepared containing 2.5 µl of 10× buffer, 0.5 µl of 10 mM mixture of dNTPs (containing 2.5 mM of each of dATP, dGTP, dCTP and dTTP) and 1.7 µl of 25 mM $MgCl_2$ in a thin walled microcentrifuge tube. Primers IVA-F, MG-F and LIS-F, 1µl of MONO-5 and MONO-7 (0.5 µl of each) and LIS-R (1.7 µl) were then added to the microcentrifuge tube (see Table 2.4) followed by 4.85 µl of sterile RO water. Ten micro-litres of extracted DNA (Section 2.10.3) and 0.25 µl of Taq DNA polymerase was then added to the PCR mixture. The tubes were then centrifuged before being placed in a thermocycler. The parameters used for the PCR reaction were initial denaturation of 95 ^{o}C for 5 min. This was followed by five cycles of 95 ^{o}C for 45 s (denaturation), 53 ^{o}C for 45 s (annealing) and then 72 ^{o}C for 45 sec (extension). This was followed by 20 cycles of 95 ^{o}C for 45 sec (denaturation), 58 ^{o}C for 45 sec (annealing), and then 72 ^{o}C for 45 sec (extension). There was then a final extension step at 72 ^{o}C for 7 min. Electrophoreses of amplified product was performed on a 2 % (w/v) agarose gel (Section 2.10.5) together with standard marker (Promega).

Table 2.4: Primer sequences used in PCR for detection of *Listeria*

Target Organism	Primer Name	Sequence (5'---> 3')	Gene target
L. monocytogenes	MONO-5-F	GCTAATACCGAATGATAAGA	16S rRNA
	MONO7- Fa	GGCTAATACCGAATGATGAA	16 S rRNA
***Listeria* genus**	LIS-R	AAGCAGTTACTCTTATCCT	16 S rRNA
	IVA-F	AGCTTGCTCTTCCAATGT	16 S rRNA
	MG-F	GCTTGCTCCTTTGGTCG	16 S rRNA
	LIS-F	AGCTTGCTCTTCCAAAGT	16 S rRNA

2.10.5 Preparation of Agarose Gels and Electrophoresis of DNA

The agarose powder (Melford Laboratories Ltd., Ipswich, UK) was mixed with 1× TAE buffer (40 mM Tris-acetate, and 1 mM EDTA [pH 8.0]) to give the appropriate final concentration of gel and was fully dissolved by microwave heating for 3 min. The agarose was cooled to about 55 °C, and 1 µl Ethidium bromide added to a final concentration of 0.5 µg ml^{-1}. The molten agarose was then poured into a gel tray containing a comb to form the wells and allowed to set. DNA samples (approx. 25 µl) were mixed with 8 µl of loading dye. A sample (4 µl) of 1 kbp ladder (Promega) was mixed with 1 µl loading dye (http://www.bioron.net/en/products/dna-and-dna-markers/dna-marker/loading-buffer-iv-6x/) and was used as a reference marker. Gels were then electrophoresed in 1 × TAE buffer in gel tank, at a voltage of 70-80 V for 30-

60 min. DNA bands were photographed using molecular imager [(Bio-Rad) integrated with Quantity One 1-D Analysis Software (Bio-Rad), Hemel Hempstead, UK].

CHAPTER 3

OPTIMIZATION AND EVALUATION OF DIFFERENT VIRUCIDAL AGENTS FOR USE IN THE PHAGE DETECTION ASSAY

3.1 Introduction

Phage detection assays are considered amongst the easiest methods used for harnessing the host specificity of phage (De Siqueira *et al*., 2003) and in particular the phage amplification assay has been successfully developed for a range of different bacteria. Hence the extension of the assay principle makes it a very promising candidate to develop a simple test to detect the presence of low levels of viable *Listeria* cells in food samples. The most crucial element for development of this type of phage-based assay is identifying a virucide that can allow rapid and efficient inactivation of the phage (De Siqueira *et al*., 2003). It is also essential that the virucide does not affect the viability of the cells being detected.

In this Chapter, the focus was on two bacteriophage (ATCC 23074-B1 and A511), which are known to have significantly different host ranges. ATCC 23074-B1 was used in an earlier work and it had been shown that the phage amplification method could be effectively performed using this phage (Dr C. Rees, University of Nottingham, unpublished data) but this is known to be a temperate phage with a limited host range and therefore this would act as a control sample (Brüssow, 2001). A511 was chosen as it is known to have a

broad host range (Loessner, 1997) but had not been used for the phage amplification format but was a good candidate to develop a phage based detection method. Hence the aim of the work presented in this Chapter was to prepare high titre stocks of these phage and also to evaluate different virucidal agents that could be used to develop a phage detection assay using phage A511.

3.2 Results

The traditional plaque assay is the "gold standard" for determining phage titres. However, the ability of the phage to infect a host strain may be significantly influenced by various factors, including the presence of bacteria, moulds and insoluble debris in the phage stock. Therefore before starting developing an assay it was important to generate large amounts of pure phage stocks that could be used as a consistent reagent throughout the rest of the project.

This experiment was designed to develop methods to produce large amounts of phage and various approaches were assessed to maximise bacteriophage growth. To scale up the lysate, different concentrations of stock phage were used in two different media (BHI or TSB) and various amounts of the lysate were then obtained between 10^5 to 10^9 plaque forming units. The bacteriophage were then propagated and concentrated by using PEG precipitation and centrifugation (Section 2.3.7). Hence, using the propagated and concentrated bacteriophage, several different chemical treatments against *Listeria* phage

(e.g. 23074-B1 and A511) as well as the host bacteria cells, were evaluated as potential virucides to achieve differential inactivation of the phage.

3.2.1 Titration and Propagation of 23074-B1 and A511

L. monocytogenes WSLC 1042 was used as the propagating strain for both phage. To prepare liquid lysates of phage, this was grown overnight in BHI broth and then diluted 1:50 into fresh BHI broth. The phage (either 23074-B1 or A511) were added to this using different multiplicities of infection (m.o.i. = 0.01, 0.1 and 0.5 for 23074-B1 and 0.01, 0.1, 0.5 and 1.0 for A511) to try and determine the best cell to phage ratio to produce good cell lysis in liquid culture. The infected cultures were incubated at 37 °C with continuous shaking (~200 rpm) in an orbital shaker. Growth was monitored (OD_{600nm}) every 45 min and lysis of the cell suspension was detected when a reduction in optical density was observed, indicating that the phage had infected and lysed the host cells.

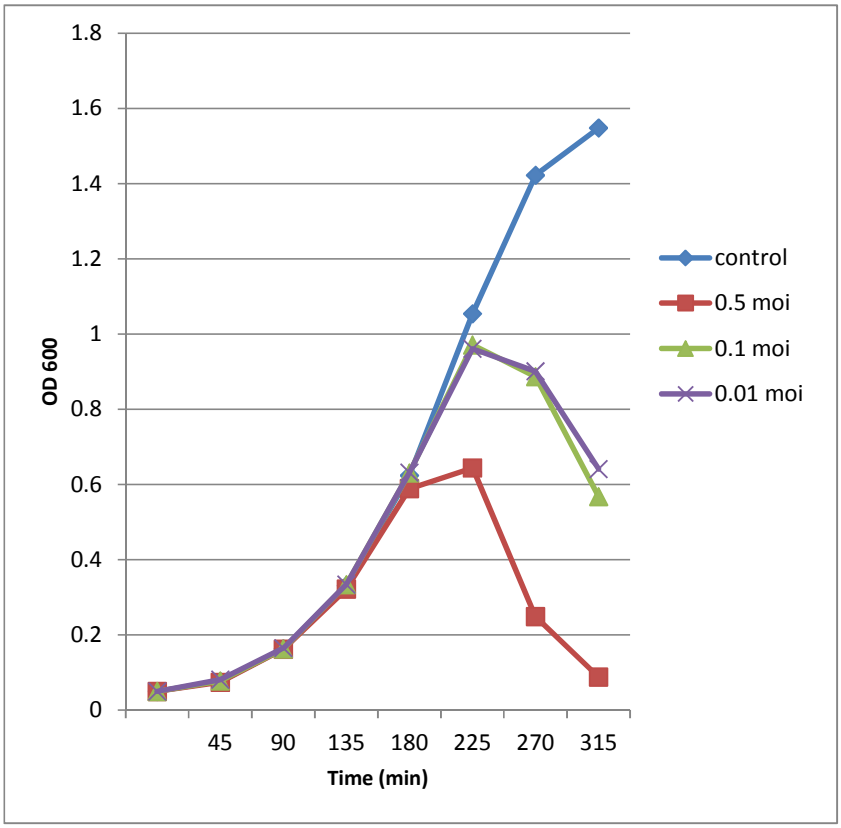
For the two phage it was seen that the OD_{600nm} values noticeably decreased at slightly different times (225 min for 23074-B1 and 135 min for A511; respectively Figures 3.1 and 3.2). The fact that 23074-B1 could be propagated in liquid cultures was useful, as many temperate phage do not replicate well in liquid and often have to be prepared by recovering phage from agar overlays.

It was also noted that when a higher ratio of phage (m.o.i) was used, cell lysis occurred earlier than when using the lower m.o.i values. This was as expected, and suggested that using a lower m.o.i. would be more productive since the cell culture had grown to a higher number before the phage population infected and

lysed all of the cells. As each infected cell produces more phage, the higher the OD of the culture before lysis occurs the more phage should be recovered.

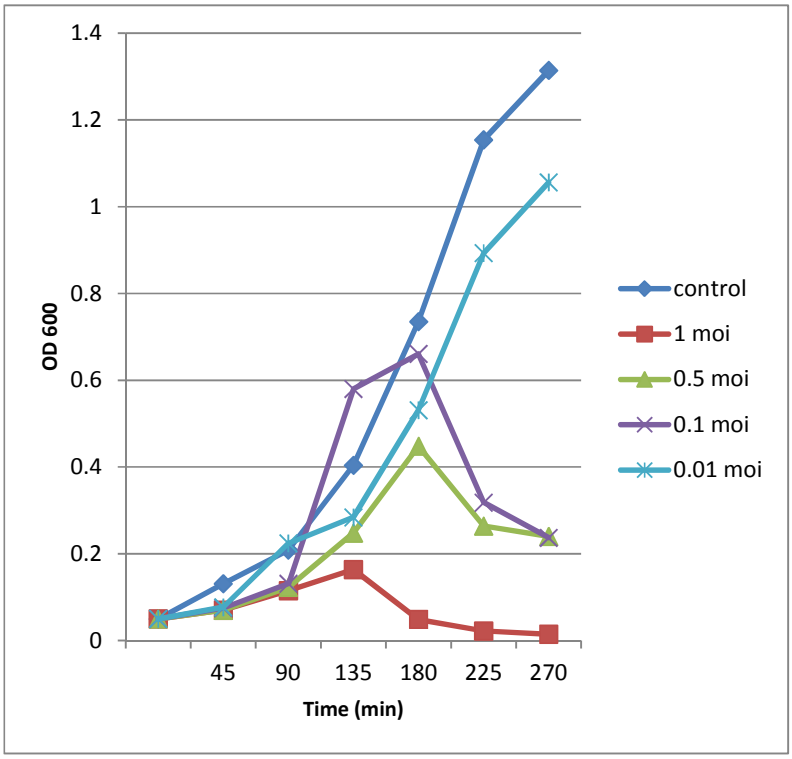
The titre of the phage lysates was determined by using the phage titration method (Section 2.3.5). However very few plaques could be seen in the 23074-B1 experiment. It was found that the plaque growth of the temperate phage was difficult to see when the lawns of *L. monocytogenes* WSLC 1042 grew too quickly. To address this, the plates were incubated at 30 °C for 18 – 24 h instead of 37 °C and plaque growth was then more visible. Using this method the phage titre obtained for the liquid culture experiment was 7.0×10^9 pfu ml^{-1} for 23074-B1. For good visualisation of plaques produced by A511 it was found that inoculating single colonies of *L. monocytogenes* WSLC 1042 into 20 ml TSB instead of BHI and then incubating the cultures at 37 ºC with continuous shaking (e.g. 200 rpm) overnight (Table 3.1) produced the best result and using this method the highest titre phage stock for A511 (e.g. 1.3×10^9 pfu ml^{-1}) was achieved.

Figure 3.1 Effect of phage 230741-B1 on growth of *L. monocytogenes* grown in BHI



One representative experiment of three independent experiments, after adding phage 23074-B1 (0.01, 0.1 and 0.5, m.o.i) to the culture of *L. monocytogenes* WSLC grown in BHI at an initial OD_{600nm} = 0.05. OD_{600nm} was monitored every 45 min and after 225 min this began to decrease. After 6 h complete lysis of the bacterial cells was obtained using the highest m.o.i.

Figure 3.2 Effect of phage A511 on growth of *L. monocytogenes* grown in TSB



One representative experiment of three independent experiments, after adding phage A511 (0.01, 0.1, 0.5 and 1.0, m.o.i) to the culture of *L. monocytogenes* WSLC grown in TSB at an initial OD_{600nm} = 0.05. OD was monitored every 45 min and after 135 min this began to decrease. After 4 h complete lysis of the bacterial cells was obtained using the highest m.o.i.

Table 3.1 Effect of different m.o.i.s of *Listeria* phage A511 and 23074 B1 on the growth of *L. monocytogenes* WSLC.

Phage 23074-B1		**Phage A511**	
m.o.i.	**Number of plaques detected** (pfu ml^{-1})	**Number of plaques detected** (pfu ml^{-1})	**m.o.i.**
0.01	1.6×10^7	3.0×10^3	0.01
0.1	1.3×10^9	2.1×10^7	0.1
0.5	7.0×10^9	1.3×10^9	0.5

One representative experiment of three independent experiments, *these results demonstrated that both phage 23074-B1 and A511 were now of a sufficient titre and can be used both phage for purification and concentration of phage.*

3.2.2 Bacteriophage Concentration and Purification

Once the best parameters for phage propagation had been determined these were used for large scale phage growth. Phage 23074-B1 or A511 (0.5 ml; 1.0 $\times 10^9$ pfu ml^{-1}) were added to 1000 ml of (O/N) culture of *L. monocytogenes* that had been diluted to $OD_{600nm} \approx 0.05$ and incubated in an orbital shaker at 37 ºC until lysis of the cells was clearly visible (approx. 5 h). Phage suspensions were then centrifuged to remove cell debris and any remaining intact cells and 10 % (w/v) of PEG 8000 added to precipitate the phage (Section 2.3.7). Samples were stored overnight at 5 °C, and phage recovered by centrifugation. Pellets containing phage were resuspended in 20 ml Lambda buffer ($1/40^{th}$ of

initial culture volume) and the titre of the phage was determined (Section 2.3.5). The final titres of the phage were 3.4×10^9 pfu ml^{-1} for 23074-B1 and 6.0×10^9 pfu ml^{-1} for phage A511. These phage stocks were then used for the evaluation of the activity of virucide that might be used in the phage detection assay.

3.2.3 Evaluation of the Activity of Virucide

To identify a suitable virucide, the virucide activity of a wide range of chemical treatments was evaluated for both phage 23074-B1 and A511. As it is essential for the assay format that the virucide causes the destruction of all bacteriophage that have not infected the host cells without affecting the viability of the target bacterial cells, the chemicals were also used to challenge the *Listeria* cells. The chemicals chosen were acetic acid, ferrous sulphate, ferrous ammonium sulphate (Virusol), hydrogen peroxide and tea infusions (De Siqueira *et al*. 2006) which had all previously been used when developing phage amplification assays (Dr C Rees, pers. comm.). De Siqueira

To test for inactivation of the phage by the virucide, a quantity (100 µl) of the phage (10^8 pfu ml^{-1} B1 and 10^9 pfu ml^{-1} A511) was added to 100 µl of Lambda buffer in a sterile Eppendorf tube, and this was treated with 100 µl of virucide and incubated 20 min at room temperature. The titre of the phage suspension was then determined before and after addition of the virucide (Section 2.3.5).

To test for antimicrobial effects of the virucide on the cells, 1 ml of *L. monocytogenes* WSLC 1042 culture (Section 2.1.17). Aliquots of this cell suspension (100 µl) were transferred to sterile Eppendorf micro-centrifuge tubes. Cells were treated with 100 µl of virucide and incubated for 20 min at room temperature and the viable count of the cells determined before and after treatment.

3.2.3.1 Treatment with Acetic Acid

To test acetic acid, the cells and phage were initially treated with 100 µl of acetic acid solutions (0.31 % or 0.62 % or 0.93 %) as described in Section 2.1.4. The results (Table 3.2) of the effect of acetic acid showed that there was a small reduction in the viability of the host strain using all 3 concentrations of virucide but the bacteria were considered to be reasonably resistant to acetic acid but even a small antimicrobial effect was not acceptable.

The results obtained from the phage challenge test are presented in Table 3.3. It was noticed that for all the concentrations examined, the results showed less than 1 $\log_{10}$ reduction in phage titres for both A511 and 23075-B1. Therefore, it is clear that CH_3COOH did not destroy either of the different phage types efficiently, and that phage are resistant to acetic acid. Hence acetic acid was not seen as an appropriate virucide.

Table 3.2 Sensitivity of bacterial cells of *L. monocytogenes* WSLC to different concentrations of acetic acid solutions

Concentration	Number of cells cfu ml^{-1}	% survival	Control (without treatment)
0.31%	3.6×10^7 2.5×10^7 4.7×10^7	32 ± 9.74	1.13×10^8 cfu ml^{-1}
0.62%	1.8×10^7 1.1×10^7 2.5×10^7	16 ± 6.20	
0.93%	2.8×10^7 3.8×10^7 1.8×10^7	25 ± 8.85	

The method used to treat the cells with the virucides is as previously described in Section 2.1.4. Results used in this table were the average of three replicates.

Table 3.3 Sensitivity of phage 23074-B1 and A511 to different concentrations of acetic acid solutions

Concentration Treatment		23074-B1		A511	
		Number of plaques (pfu ml^{-1})	% survival	Number of plaques (pfu ml^{-1})	% survival
0.31%	**before**	2.4×10^8 2.1×10^8 2.5×10^8	87 ± 40.1	1.6×10^9 2.2×10^9 8.0×10^8	12 ± 8.61
	after	1.0×10^8 2.1×10^8 3.0×10^8		3.4×10^8 1.8×10^8 4.0×10^7	
0.62%	**before**	2.3×10^8 4.1×10^8 5.7×10^8	69 ± 62	6.0×10^8 2.8×10^9 1.9×10^9	22 ± 31.7
	after	3.2×10^8 2.2×10^8 1.0×10^8		3.5×10^8 1.9×10^8 1.0×10^7	
0.93%	**before**	7.5×10^8 5.6×10^8 3.1×10^8	18 ± 5.11	2.2×10^9 4.5×10^9 1.8×10^8	39 ± 48.4
	after	1.1×10^8 9.1×10^7 7.5×10^7		2.5×10^8 3.7×10^8 1.7×10^8	

The method used to treat the cells with the virucides is as previously described in Section 2.1.4.

Results used in this table were the average of three experiments.

3.2.3.2 Treatment with Virusol

The Virusol used in this study was that supplied in the commercial *FASTplaque*TB™ kit and consists of 10 mM ferrous ammonium sulphate, which has been used for a phage amplification assay for detection of *Mycobacterium* as described by Stanley *et al*. (2007).

A quantity (100 µl) of Lambda buffer was mixed with 100 µl of the phage (titre of phage used were 10^8 pfu ml^{-1} and 10^9 pfu ml^{-1} for 23074-B1 and A511, respectively) which was treated with 100 µl Virusol prepared according to the manufacturer's instructions. The Virusol was also tested at a 50 % concentration (5 mM) using the same method used above. Both treated phage samples were incubated at room temperature for 20 min.

The results showed that Virusol has slightly reduced the titre of phage by approx. 1 $\log_{10}$ (Table 3.4). Therefore, it seems most likely that the phage are also resistant to Virusol and, therefore, cannot be used in this experiment for a phage detection assay that allows rapid detection of *Listeria* in food.

Table 3.4 Sensitivity of phage 23074-B1 and A511 to Virusol

23074-B1				**A511**	
Virucide	**Stage of Treatment**	**Number of plaques (pfu ml^{-1})**	**Survival %**	**Number of plaques (pfu ml^{-1})**	**Survival %**
Virusol*	Before	3.7×10^7 5.9×10^7 7.6×10^7	34 ± 30	7.0×10^8 9.0×10^8 5.0×10^8	14 ± 4.7
	After	2.7×10^7 1.4×10^7 5.0×10^6		8.7×10^7 8.8×10^7 9.5×10^7	
50% Virusol**	Before	7.6×10^8 5.6×10^8 3.6×10^8	7 ± 5.18	1.1×10^9 2.1×10^9 1.1×10^9	48 ± 28
	After	3.7×10^8 2.9×10^8 1.5×10^8		6.0×10^8 2.5×10^8 8.0×10^8	

** 10 mM Ferrous Ammonium Sulphate. **5 mM Ferrous Ammonium Sulphate. Results used in this table were the average of three experiments.*

3.2.3.3 Treatment with Ferrous Sulphate and Hydrogen Peroxide

The challenge experiment was extended to include other potential chemicals, such as ferrous sulphate and hydrogen peroxide. In this experiment phage and cells were treated with 100 µl of ferrous sulphate (4.3 mM) or hydrogen peroxide (3.5 %) (Stewart *et al*., 1998), and the treated samples were again incubated at room temperature for 20 min. Hydrogen peroxide at 3.5 % had a significant effect on the viability of the *Listeria* cells, as the viable count was reduced by 8 $\log_{10.}$ Therefore, it can be concluded that *L. monocytogenes* WSLC 1042 is sensitive to hydrogen peroxide; and hence, this reagent also cannot be used for a phage detection assay (Table 3.5). Since the effect of H_2O_2 on the cells was so dramatic, it was not used to challenge the phage.

The effect of the ferrous sulphate solution was similar to that seen with acetic acid, showing a small reduction in cell viability which was not desired. Also Table 3.6 shows that about $1\log_{10}$ reduction in phage A511 titre was obtained while Phage 23074-B1 seemed to be slightly more resistant to ferrous sulphate. Hence, this reagent was also excluded as a potential virucide in this experiment. Based on the results presented above, ferrous sulphate or hydrogen cannot be used in this experiment to develop a phage detection assay.

Table 3.5 Sensitivity of bacterial cells of *Listeria monocytogenes* WSLC to ferrous sulphate and hydrogen peroxide

Treatment	Number of cells (cfu ml^{-1})	Survival %	Control (without treatment)
Ferrous sulphate 4.3 mM	6.6×10^8 4.5×10^8 3.3×10^8	12 ± 4.07	4.1×10^9 cfu ml^{-1}
Hydrogen peroxide 3.5 %	0	0	

The method used to treat the cells with the virucides is as previously described in Section 2.1.5 and section 2.1.6. Results used in this table were the average of three replicates.

Table 3.6: Sensitivity of phage 23074-B1 and A511 to ferrous sulphate

Treatment	23074-B1		A511	
	Number of plaques (pfu ml^{-1})	Survival (%)	Number of plaques (pfu ml^{-1})	Survival (%)
Before	8.5×10^7 7.4×10^7 9.6×10^7	35 ± 16	5.6×10^9 4.4×10^9 2.0×10^9	5.0 ± 1.6
After	2.9×10^7 4.1×10^7 1.7×10^7		2.0×10^8 3.0×10^8 1.0×10^8	

The method used to treat the phage with the virucides is as previously described in Section 2.1.5. Results used in this table were the average of three experiments.

3.2.3.4 Treatment with Tea Infusions

Treatment with tea infusion was carried out based on the observations as described by De Siqueira *et al*. (2006) who had shown that tea extracts could be used as a virucide for the phage amplification assay. Eight types of loose-leaf tea were used and the tea infusions were prepared as described in Section 2.1.7. In order to assure the sterility of the tea infusion, samples (10 µl) of each tea infusion were spotted onto BHI agar plates and incubated at 30 °C for 18 – 24 h to confirm that there was no significant cell growth.

The tea infusions were then screened for virucidal activity against the phage. It was found that the tea infusions showed a good ability to inhibit the infectivity of phage (e.g. 23074-B1 and A511). Depending on the tea sample used approximately 4, 5, 6 or 8 $\log_{10}$ reductions in viable phage numbers were observed when treating phage 23074-B1 (Table 3.7), and about 9-$\log_{10}$ reduction in phage titres were observed when phage A511 were treated in all samples (Table 3.8). From these results, it can be seen that Thyme did not affect the phage and therefore this type of herb extract was not effective. Phage A511 was found to be more sensitive to the virucide treatment than phage 23074-B1 and tea infusions were found to be good virucidal agents and, therefore, they were potential agents that could be used to further develop a phage detection assay.

Table 3.7 Sensitivity of phage 23074-B1 to various tea infusions

Samples	Treatment	number of plaques (pfu ml^{-1})	Samples	Treatment	number of plaques (pfu ml^{-1})
Green tea, Gunpowder Leaf Tea	**B**	5.4×10^8 6.1×10^8 4.7×10^8 $5.4\times10^8 \pm 5.7\times10^7$ 5.3×10^8	**DARJEELING Leaf Tea**	**B**	1.6×10^8 1.5×10^8 8.3×10^7 $1.5\times10^8 \pm 6.0\times10^7$ 2.3×10^8
	A	Nil		**A**	2.5×10^2 2.4×10^2 2.6×10^2 $2.5\times10^2 \pm 9.5$ 2.6×10^2
Green tea, Sencha Leaf Tea	**B**	1.0×10^8 1.5×10^8 5.2×10^7 $1.96\times10^8 \pm 1.94\times10^8$ 4.8×10^8	**Earl Grey, Leaf Tea**	**B**	7.2×10^7 9.7×10^7 5.2×10^7 $7.4\times10^7 \pm 1.8\ 2\times10^7$ 7.5×10^7
	A	Nil		**A**	1.2×10^3 1.3×10^3 1.1×10^3 $1.2\times10^3 \pm 1.4\times10^2$ 9.5×10^2
ASSAM, Leaf Tea	**B**	8.9×10^7 7.5×10^7 1.0×10^8 $8.8\times10^7 \pm 1.0\times10^7$ 9.1×10^7	**Organic Green Loose Leaf Tea**	**B**	2.6×10^7 3.8×10^7 1.4×10^7 $2.6\times10^7 \pm 9.8\times10^6$ 2.5×10^7
	A	5.7×10^2 6.8×10^2 6.5×10^2 $5.9\times10^2 \pm 9.3\times10^1$ 4.7×10^2		**A**	1.4×10^3 1.7×10^3 1.2×10^3 $1.3\times10^3 \pm 2.9\times10^2$ 1.0×10^3
Ceylon, Leaf Tea	**B**	1.2×10^9 1.8×10^9 6.3×10^8 $1.2\times10^9 \pm 4.8\times10^8$ 1.3×10^9	**Kenya Leaf Tea**	**B**	6.5×10^7 6.4×10^7 1.1×10^8 $6.6\times10^7 \pm 3.4\times10^7$ 2.5×10^7
	A	2.9×10^4 4.0×10^4 1.8×10^4 $2.9\times10^4 \pm 9.0\times10^3$ 2.8×10^4		**A**	7.0×10^3 9.0×10^3 5.0×10^3 $7.1\times10^3 \pm 1.6\times10^3$ 7.7×10^3
Thyme	**B**	8.5×10^7 6.4×10^7 1.1×10^8 $8.5\times10^8 \pm 1.8\times10^7$ 8.4×10^7			
	A	1.0×10^7 1.1×10^7 1.6×10^7 $1.0\times10^7 \pm 4.7\times10^6$ 4.5×10^6			

The method used to treat the phage with the virucides is as previously described in section 2.1.7. Results used in this table were the average of four replicates . (A) After, (B) Before.

Table 3.8 Sensitivity of phage A511 to various tea infusions

Samples	Treatment	number of plaques (pfu ml^{-1})	Samples	Treatment	number of plaques (pfu ml^{-1})
Green tea, Gunpowder Leaf Tea	**B**	2.0×10^9 1.6×10^9 2.3×10^9 $2.0 \times 10^9 \pm 2.9\times 10^8$ 2.1×10^9	**DARJEELING, Leaf Tea**	**B**	5.2×10^9 4.9×10^9 2.8×10^9 $4.1 \times 10^9 \pm 1.1\times 10^9$ 3.8×10^9
	A	Nil		**A**	Nil
Green tea, Sencha Leaf Tea	**B**	2.9×10^9 3.5×10^9 2.7×10^9 $3.0\times 10^9 \pm 3.4\times 10^8$ 3.0×10^9	**Earl Grey, Leaf Tea**	**B**	3.8×10^9 4.9×10^9 2.8×10^9 $3.5\times 10^9 \pm 8.7\times 10^8$ 3.5×10^9
	A	Nil		**A**	Nil
ASSAM, Leaf Tea	**B**	3.8×10^9 4.8×10^9 2.8×10^9 $3.7\times 10^9 \pm 8.1\times 10^8$ 3.7×10^9	**Organic Green Loose Leaf Tea**	**B**	5.1×10^9 6.3×10^9 4.7×10^9 $3.5\times 10^9 \pm 6.8\times 10^8$ 5.3×10^8
	A	Nil		**A**	Nil
Ceylon, Leaf Tea	**B**	6.2×10^9 7.2×10^9 5.0×10^9 $6.2 \times 10^9 \pm 9.0\times 10^8$ 6.4×10^9	**Kenya, Leaf Tea**	**B**	5.3×10^9 4.8×10^9 2.8×10^9 $4.5\times 10^9 \pm 1.1\times 10^9$ 3.9×10^9
	A	Nil		**A**	Nil
Thyme	**B**	4.5×10^9 5.3×10^9 3.7×10^9 $4.5 \times 10^9 \pm 6.0\times 10^8$ 4.6×10^9			
	A	1.0×10^8 1.5×10^8 5.3×10^7 $1.9\times 10^8 \pm 1.8\times 10^8$ 4.7×10^8			

The method used to treat the phage with the virucides is as previously described in section 2.1.7. Results used in this table were the average of four replicates. (A) After, (B) Before.

3.2.3.5 Effect of Freezing Tea Infusions

Since the aim was to develop a practical assay, it was seen that there was a need to produce large amounts of the tea infusion that could be stored for a long period of time to allow experiments to be performed reproducibly. Therefore three types of tea leaves were chosen to test antimicrobial activity on bacterial cells after the tea infusion had been frozen. Tea infusions were frozen by placing aliquots of 1 ml at –20 °C overnight. Samples were then thawed by holding in the hand for 5 min to thaw.

The results of sensitivity of cells using both fresh and frozen samples of virucide on cells are presented in Tables 3.9. The results showed that tea infusion, prepared from various types of tea leaves did have some effect on the viability of bacteria tested but the Gun Powder Leaf tea after freezing seemed to have no affect on cell viability at all. Therefore, the bacteria were resistant to this type of tea infusion after the freezing treatment.

Table 3.9 Effect of freezing of tea infusion on *L. monocytogenes* cells

Sample	Treatment	Replicate Experiments			Average
		Log cfu ml $^{-1}$ 1	Log cfu ml $^{-1}$ 2	Log cfu ml $^{-1}$ 3	
Green tea, Gunpowder Leaf Tea	Before freezing	8.662	8.623	8.748	8.678 ± 0.052
	After freezing	9.462	9.491	9.397	9.450 ± 0.039
Green tea, Sencha Leaf Tea	Before freezing	8.342	8.113	8.477	8.311 ± 0.149
	After freezing	8.819	8.857	8.681	8.786 ± 0.075
ASSAM, Leaf Tea	Before freezing	8.8	8.230	7.361	7.864 ± 0.367
	After freezing	8.662	8.903	8.056	8.540 ± 0.356

The method used to treat the cells with the virucides is as previously described in section 2.1.7. Results used in this table were the average of three experiments.

The results shown in Table 3.10 show the effect of freezing tea infusion on phage inactivation and indicate that for 23075-B1 5, 4, 3 $\log_{10}$ reductions in phage titre were achieved using Gunpowder Leaf Tea, Sencha Leaf and ASSAM Leaf Tea, respectively. In contrast phage A511 were more sensitive to the virucide treatment and no viable phage could be detected after treatment and so this was found to be a good virucidal agent. In general, the results demonstrate that little change has occurred in the tea infusion activity after freezing of tea infusions, although their effect on phage 23074-B1 became less effective.

Table 3.10 Effect of freezing tea infusion on Phage 23074-B1 and A511

Samples	Treatment	23074-B1 number of plaques (Log pfu ml^{-1})	A511 number of plaques (Log pfu ml^{-1})
Green tea, Gunpowder Leaf Tea	Before Treatment	8.60 8.69 8.59 ± 0.081 8.49	9.54 9.81 9.321 ± 0.521 8.60
	After Freezing	3.77 3.90 3.66 ± 0.256 3.30	Nil
Green tea, Sencha Leaf Tea	Before Treatment	8.78 9.11 8.55 ± 0.568 7.77	9.57 9.25 9.53 ± 0.21 9.76
	After Freezing	4.11 3.93 4.09 ± 0.121 4.23	Nil
ASSAM, Leaf Tea	Before Treatment	7.66 7.72 7.65 ± 0.059 7.57	9.14 9.36 9.08 ± 0.257 8.74
	After Freezing	4.20 4.04 4.18 ± 0.107 4.30	Nil

The method used to treat the phage with the virucides is as previously described in section 2.1.7. Results used in this table were the average of three experiments.

The efficiency of tea infusion was tested by adding different concentrations of Gunpowder Leaf Tea to the phage A511. At low concentrations the effect of the tea was sub-optimal as incomplete phage inactivation was recorded. However, when the concentrations of tea were increased, the efficiency increased (Table 3.11).

Table 3.11 Sensitivity of phage A511 to different concentrations of tea infusion

Concentration of tea infusion	No. of plaques (Log pfu ml^{-1})
*2.0 %	5.20 5.30 5.18 ± 0.107 5.04
3.0 %	4.47 4.65 4.435 ± 0.196 4.17
4.0 %	0
5.0 %	0
6.0 %	0
7.0 %	0

*2.0 % tea infusion (w/v)

The method used to treat the phage with the virucides is as previously described in section 2.1.7. Results used in this table were the average of three experiments.

3.3 Discussion

When titering phage stocks it was clear that efficient infection of host cells was not always occurring. This phenomenon might be because of the different nutritional and environmental conditions affecting the growth and cell surface of the cells. As a result, this can affect the formation and size of bacteriophage plaques. In this context, Lillehaug (1997) and Los *et al*. (2007) suggested that processes of plaque formation by different groups of bacteriophage may depend on different factors, such as the volume and softness of the top and bottom layers, and the number and growth stage of the bacterial cells added to the lawn, and also affect the plaque size. In this case the growth of the host

cells was found to have most effect so the titration method was modified accordingly.

The most crucial element for development of this type of phage-based assay is the inactivation of the phage by the virucide (Stewart *et al*., 1995; Stewart *et al*., 1998). It is essential that the virucide does not affect the viability of the cells being detected but achieves rapid and efficient inactivation of the phage. Optimisation of virucidal treatment is an important step which is required before a phage detection assay can be developed. In order to gain more insights into relationship between the virucidal agents, a wide range of compounds have been screened and tested in this work (e.g. acetic acid, Virusol, ferrous sulphate, hydrogen peroxide and tea infusions). Compounds that did not affect the bacterium were also screened for virucidal activity against the phage proposed for this study.

In an earlier study, Stewart *et al.* (1995, 1998) have reported that the bacteriophage can be protected by chemical and physical agents that would destroy the free bacteriophage. In this study, the sensitivity of the bacterial cells and bacteriophage B1 and A511 to ferrous sulphate (4.3 mM) and hydrogen peroxide (3.5 %) was also examined. The *L. monocytogenes* cells were very sensitive to hydrogen peroxide treatment. Similar results have been obtained and reported by Zameer and Gopal (2010), the researchers found that a hydrogen peroxide solution (3 %) could reduce the initial concentration of *Listeria* cells in biofilms from 2.57 x 10^9 cfu ml^{-1} by 4.0 $\log_{10}$ cfu ml^{-1} after 10 min of exposure, and a hydrogen peroxide solution (3.5 %) could reduce a planktonic population by 3 $\log_{10}$ and complete elimination of cells occurred

after 10 min of exposure. They also concluded that a higher concentration of hydrogen peroxide was capable of eliminating both planktonic at 3.5 % and 6 % for biofilms cells of *L. monocytogenes* (Zameer and Gopal, 2010).

The sensitivity of both bacteriophage B1 and A511 was also examined to tea and thyme (*Thymus vulgaris*) infusions. The results obtained from these experiments show that A511 was very sensitive to almost all types of tea infusions, with a maximum of 9 $\log_{10}$ reduction in pfu ml^{-1} observed. Therefore, the results demonstrate that these tea infusions were successfully able to eliminate A511. Other researchers have reported that teas contain polyphenols and most of the polyphenols are flavanols (Sakanaka *et al*., 1989; Ahn *et al*., 1991; Makhtar *et al*., 1994; Kuroda and Hara, 1999). Therefore, in this study, it is significant that the successful effect of tea infusions on bacteriophage (A511) probably comes from these compounds that might be having mild antimicrobial activity (Stewart *et al*., 1995) and active compounds do not seem to be present in the Thyme tea infusions which come from a different family of plants.

These results suggest that green tea infusions were a good virucidal agent for phage detection assay. Similar results were reported by De Siqueira *et al*. (2006). In their study, the researchers have examined nine types of loose-leaf tea as a virucidal, been used against two phage, (e.g. Felix-01 and P22) in a phage-based *Salmonella* amplification assay. They found that the extracts of these loose-leaf teas were sufficiently potent to eliminate phage Felix-01, and they also suggested using them as virucidal for the phage detection assay.

The problem with choosing plant extracts as a basis of the assay is that they are unstable for long term storage and of a variable composition. Therefore, it was important to know if they could be preserved after preparation to help with reproducibility of future research. The results of sensitivity tests using these frozen samples of virucide for both *L. monocytogenes* WSLC cells and phage demonstrate that the virucidal effect on phage A511 was not affected and no increase in antimicrobial activity was seen against the host cells.

Because of the variability that might occur when preparing tea extracts, the efficiency of Gunpowder leaf tea was tested by adding different concentrations of tea infusion to the phage A511 to determine how crucial the concentration of tea was. From data obtained it was noted that when using more than 4 % of tea infusions the number of phage was completely reduced and eliminating all phage about producing 8 $\log_{10}$ reduction in pfu ml^{-1}. The results of this study suggesting that the A511 phage was found to more sensitive to the virucide treatment than phage B1, and tea was again found to be a good virucidal agent. A511 provides a good candidate for development of the phage-based detection assay.

CHAPTER 4

DEVELOPMENT OF A PHAGE DETECTION ASSAY FOR *LISTERIA MONOCYTOGENES*

4.1 Introduction

Listeria monocytogenes has become a well-recognised pathogenic bacterium after several food-borne outbreaks. It belongs to the *Listeria* genus, and only two out of six species belonging to this genus are considered as pathogens, (i.e. *L. monocytogenes* and *Listeria ivanovii);* whilst the other four are apparently non-pathogenic species in humans (e.g. *Listeria innocua*, *Listeria seeligeri*, *Listeria welshimeri* and *Listeria grayi)* (Arslan and Özdemir, 2008; Muller *et al*., 2010; Kumar, 2011), moreover *Listeria marthii* and *Listeria rocourtiae* were classified as new species within the genus *Listeria* (Graves *et al*., 2010; Leclercq *et al*., 2010). However, the presence of any of these species on foodstuffs has been taken as an indicator of the potential contamination of the product with *L. monocytogenes* (Gilot and Content, 2002). However as these organisms are non-pathogenic, a rapid and reliable approach for differentiation of *L. monocytogenes* from the other species of the genus is very important in food catering. When assessing methods for detection of *L. monocytogenes* in foods, the key points are rapidity, sensitivity and specificity. Traditional testing methods for the detection of *L. monocytogenes* take too long, are not very sensitive or are inappropriate for application in preventative programs, such as Hazard analysis and critical control points (HACCP) that increase sanitation

efforts to eliminate contamination (Nørrung *et al*., 1999; Poltronieri *et al*., 2009).

The phage amplification assay has been among the easiest methods and low-cost phenotypic determination of the isolates and the detection of viable organisms in foods. The principle of this technique is based on the interactions of bacteriophage and their host bacteria, which can offer rapid and accurate detection of pathogens. In the phage amplification assay, the target bacterium is detected on the basis that, after infection, the host cell is able to protect a phage from the action of a virucide. Infected bacteria are then mixed in culture with high numbers of cells which can support phage replication (termed sensor cells) and, the whole sample is plated in a soft agar overlay and incubated. Bacteriophage, then, undergo rapid cycles of infection, replication and lysis which are seen as plaques arising from each infected target cell in a lawn of confluent growth of sensor cells. The number of plaques corresponds to the number of target cells initially present and infected by the phage in the sample (Park *et al.*, 2003; Stanley *et al*., 2007).

The purpose of the work present in this Chapter is to use the tea extracts identified as virucides in Chapter 3 to develop a phage-based detection method for *L. monocytogenes*.

4.2 Phage Amplification Assays

Using the information gained in Chapter 3 was possible to devise a first assay format for the phage amplification assay. Using this it was then possible to evaluate the performance of the assay and to test different variables to see how this affected the assay performance. In particular three aspects were investigated; first factors that affect plaque formation, second whether growth media used for the cells to be detected affected the efficiency of phage infection and third, whether the assay was able to detect a wide range of different *Listeria* strains with the same efficiency.

4.3 Results

4.3.1 Development of the Phage Amplification Assay for *L. monocytogenes*

The phage amplification assay was carried out as described in Section 2.5, however using the following specific details: *L. monocytogenes* WSLC 1042 cells were 10-fold serially diluted in Lambda buffer and incubated for the time necessary to be infected by the phage A511. The phage used was 10^9 pfu ml^{-1} since this was sufficient to ensure that all cells present in a sample would be infected. The mixture was then treated by 500 µl tea infusion (9 $\log_{10}$ reduction were achieved, see Chapter 3 section 3.2.3.4) as a virucide to destroy any viruses that have not infected the host cell. Infected *Listeria* were then mixed in culture with *L. innocua* (non-pathogenic bacteria used to form the lawn) and

this was mixed with 5.0 ml of BHI soft agar (0.4 %) (Section 2.1.18) and then poured as an overlay onto a BHI agar plate (Section 2.1.8). Samples were incubated at 30 °C for 18 – 24 h. After this plaque counts were obtained as an indication of cell number.

4.3.2 Evaluation of the Phage Amplification Assay for *L. monocytogenes*

To evaluate the success of the assay, viable cell counts (Section 2.3.2) were used to determine the number of *L. monocytogenes* WSLC 1042 cells added to the assay (in this case 7.5×10^5 CFU). For the assay, the negative control sample (buffer alone) produced no plaques showing that all the phage were destroyed if no host cells were present in the sample. However plaques were detected at the end point of the assay when *L. monocytogenes* cells were added, indicating that the phage was able to infect *L. monocytogenes* WSLC 1042 cells and was protected from the action of the virucide. The sample that produced countable numbers of plaques was used to determine the number of *L. monocytogenes* cells detected and this was found to be 1.44×10^6 pfu (Table 4.1), indicating that the number of the plaque forming units detected correlated closely to the number of *Listeria* cells initially introduced into the test sample. In this experiment, the assay could detect *Listeria* at approximately a ratio of 1 pfu: 1 cfu. Images of the plaque results are shown in Figure 4.1.

Table 4.1 Detection of viable *L. monocytogenes* WSLC using phage detection assay.

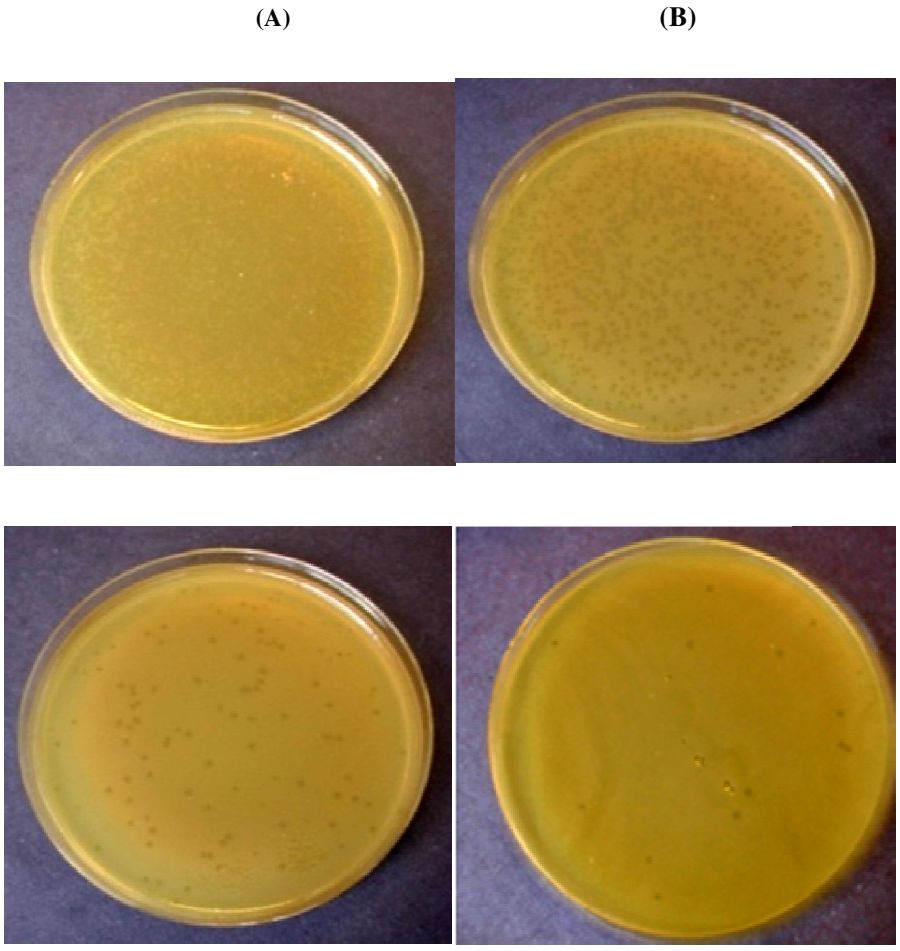
		Total number of *L. monocytogenes* cells detected using assay/ 0.5 ml	**Number of viable *L. monocytogenes* cells added to sample/** 0.5 ml
Serial Dilutions	**Number of plaques**		
10^{-2}	[a] Complete lysis		
10^{-3}	[b] TNTC		
10^{-4}	144 pfu	1.44×10^6	7.5×10^5
10^{-5}	≥10 pfu		
10^{-6}	0		
[c] **Negative Control**	0		

One representative experiment of three independent experiments.

[a] Complete lysis, lysis of all of the lawn by L. innocua. [b]TNTC, too numerous to count (more than 200 plaques per plate). [c] Control negative was 500 µl of Lambda buffer without L. monocytogenes

To prepare the inoculum *L. monocytogenes* WSLC 1042 cultures (1.5×10^7 cfu ml^{-1}; Section 2.3.2) was diluted in ten-fold steps in Lambda buffer (Section 2.1.1) and 500 µl aliquots of each dilution used for the phage amplification assay (Section 2.5). As a negative control 500 µl of Lambda buffer containing no *L. monocytogenes* cells was used.

Figure 4.1 Representative results of phage amplification assay



A culture of *L. monocytogenes* WSLC 1042 containing 1.5×10^7 cfu ml^{-1} was ten-fold diluted in Lambda buffer and a 0.5 ml sample of each dilution was tested using the phage assay as described in Section 2.5. Numbers of plaques visible on plates were (panel A) 10^{-2} dilution (confluent lysis), (panel B) 10^{-3} Too Numerous To Count (TNTC), (panel C) 10^{-4} (144 plaques) and (panel D) 10^{-5} (≤10 plaques).

4.3.3 Optimisation of Phage Assay Conditions

4.3.3.1 Optimal Soft Agar Concentration For Plaque Production

Although the initial phage assay results were encouraging, the visualisation of the plaques was difficult. This was related to the problem reported in Chapter 3 when initial phage titering was performed. To try and improve the assay different concentrations of top layer agar were used to try and improve plaque visualisation. The results from experiments to determine the viable counts of the same *L. monocytogenes* WSLC 1042 culture using top layer agar containing different percentages of agar are shown in Table 4.2.

From Table 4.2, it can be shown that the ratio of plaque number to colony count increased as the percentage of the agar decreased with the lowest concentration (0.4% soft agar) giving the highest cfu: pfu ratio (0.6).

The comparison between plaque and colony counts indicated that on this occasion the colony forming unit (cfu): plaque forming unit (pfu) ratio was not 1, and therefore some cells in the sample were still not detected. This would have the effect of reducing the limit of detection of the assay. However, since it was envisaged that this assay would be used after enrichment of samples rather than for direct detection of *Listeria* in foods (see Chapter 2, Section 2.6), this would not present a problem. The results did emphasise that the parameters affecting plaque size increased the detection limit since an increase in the size of plaques helped achieve good plaque detection.

Table 4.2: Effect of agar concentration in overlay on the detection of viable *L. monocytogenes* WSLC

Soft agar concentration	Number of *L. monocytogenes* added (cfu ml^{-1})	Number of plaques obtained (pfu $ml^{-1)}$	cfu:pfu ratio
0.4 %	5.5×10^7	3.3×10^7 3.3×10^7 3.1×10^7	1:0.587 ± 0.0231
0.5 %	5.5×10^7	3.2×10^7 1.1×10^7 5.3×10^7	1:0.58 ± 0.380
0.6 %	7.0×10^7	3.1×10^7 5.0×10^7 1.2×10^7	1:0.44 ± 0.270
0.7 %	7.5×10^7	3.1×10^7 2.0×10^7 4.2×10^7	1:0.41 ± 0.150

To prepare standard inocula for the phage assay L. monocytogenes WSLC 1042 culture (Section 2.3.2) was diluted in ten-fold steps in Lambda buffer (Section 2.1.1) and 500 µl aliquots of each dilution used for the phage amplification assay. In this case the 5 ml soft BHI agar was prepared using different amounts of agar (0.4 %, 0.5 %, 0.6 % and 0.7 %). Plates were incubated at 30 °C for 18 h. Results used in this table were the average of three replicates.

4.3.3.2 Optimal Growth on Different Media For Plaque Production

For the application of the phage detection assay to enrichment cultures, it was necessary to examine the potential influence of the growth media on the ability of the phage to infect *Listeria* cells. So far the performance of this new phage detection assay had been performed using cells that had been grown in rich laboratory media. In order to investigate this, it was necessary to determine the effect of growing this organism in different media, the defined media D10 and MCDB 202 (to represent growth of the organism in nutrient poor conditions)

and Fraser broth which is routinely used for the isolation of *Listeria* from foods.

To investigate this, *L. monocytogenes* WSLC 1042 was grown in these four different media, brain heart infusion broth (Section 2.1.8), Fraser broth (Section 2.1.10), D10 defined medium, and MCDB 202 medium (Sections 2.1.14 and 2.115) and then dilutions of each culture prepared in Lambda buffer. Samples taken from each dilution were examined using the phage detection assay, and in each case, the viable count of each sample under examination was determined.

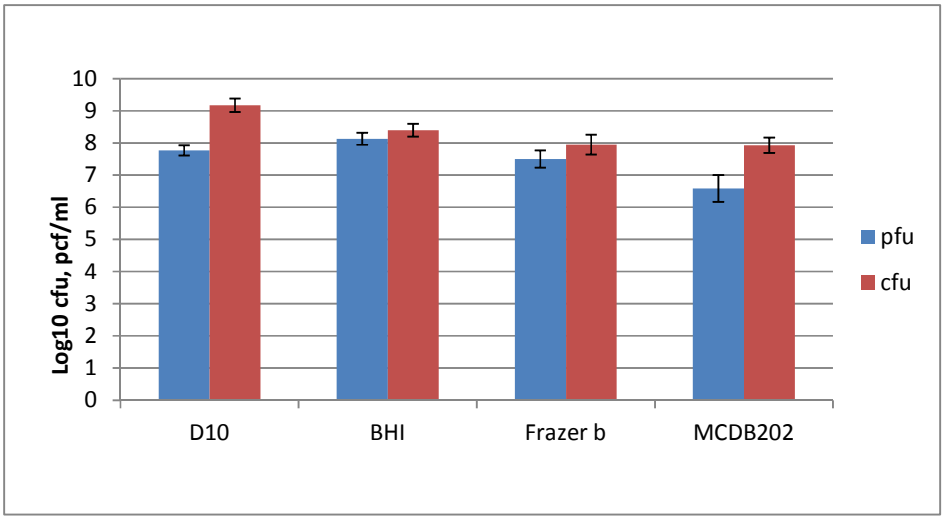
Table 4.3 and Figure 4.2 show the results obtained in this experiment. The cfu: pfu ratio for cells grown in BHI was in line with other results (1:0.5) whereas when cells were grown in D10 defined medium the cfu: pfu ratio was 1:0.04 and for MCDB 202 1:0.05 showing that in these media the phage were less able to infect the *Listeria* cells. However, when the cells were grown in Fraser broth, it can be seen that a ratio between plaque and colony count closer to that seen for BHI broth was obtained (1:0.35) and therefore the phage detection assay was most efficient when cells were grown in BHI and Fraser broth media, but suggesting that the new phage assay is suitable for the detection of *Listeria* cells after performing enrichments from food samples.

Table 4.3: Effect of growth media on the detection of viable *L. monocytogenes* WSLC

Growth media	Titre (cfu ml^{-1} or pfu ml^{-1})	Replicate Experiments			Average	cfu:pfu ratio
		1	2	3		
BHI	**cfu ml^{-1}**	1.5×10^8	3.9×10^8	2.5×10^8	2.6×10^8	1:0.34 ± 0.192
	pfu ml^{-1}	1.3×10^8	2.0×10^8	8.7×10^7	1.3×10^8	
Frazer broth	**cfu ml^{-1}**	8.9×10^7	1.8×10^8	4.3×10^7	1.0×10^8	1:0.35 ± 0.025
	pfu ml^{-1}	3.2×10^7	5.8×10^7	1.6×10^7	3.5×10^7	
MCDB 202	**cfu ml^{-1}**	8.5×10^7	1.4×10^8	4.8×10^7	9.1×10^7	1:0.05 ± 0.101
	pfu ml^{-1}	4.1×10^6	1.4×10^6	9.7×10^6	5.0×10^6	
D10	**cfu ml^{-1}**	1.4×10^9	2.3×10^9	9.3×10^8	1.5×10^9	1:0.04 ± 0.04
	pfu ml^{-1}	5.8×10^7	4.0×10^7	8.5×10^7	6.1×10^7	

The assay was performed by diluting overnight cultures of L. monocytogenes WSLC 1042 grown in different media in Lambda buffer and preparing ten-fold dilutions. A 500 µl sample of each dilution was then tested using the phage amplification method described in Section 4.3.1 using 0.4 % BHI top layer agar. Viable count was determined as described in Section 2.3.5.

Figure 4.2: Effect of different growth media on detection of viable *L. monocytogenes* WSLC using the phage detection assay



This assay was performed by diluting overnight cultures of *L. monocytogenes* WSLC 1042 (~10^8 cfu ml^{-1} see Table 4.3) in Lambda buffer. 500 µl of each dilution was transferred into a sterile reaction vessel and mixed by shaking for 3 min with 100 µl phage (10^8 pfu ml^{-1} A511). The samples were shaken and then incubated at 37 °C for 60 min. Virucide solution (500 µl of tea infusion; Section 2.1.7) was added to the sample mixture, and then incubated at room temperature for 20 min with continuous shaking. Finally 100 µl of an overnight culture of *L. innocua* (Section 2.3.2) was mixed with 5 ml of BHI (0.4 %) top layer agar, poured onto BHI agar and incubated at 30 °C for 18 h. *Results used in this Figure were the average of three experiments.*

4.3.4 Detection of Different Serotypes of *Listeria* spp. Using the Phage Assay

The phage assay was initially developed using the phage propagating strain of *L. monocytogenes* WSLC 1042 (serovar 4b). Hence the assay was further evaluated using several different isolates of *Listeria*, representing other serotypes commonly associated with food poisoning demonstrating that all these strains could be detected using the phage assay.

The strains of *Listeria* chosen were based on the serovar, and included some well studied *L. monocytogenes* strains such as EGDe (serotype 1/2 a), ATCC

23074 (serotype 4b), 10403S (serotype 1/2 a) and some isolates held in the laboratory collection that had been isolated from either food or milk (see Table 2.2). Strains chosen represented different serotypes and were Lm 1 (serotype 1/2 a), Lm 3 (serotype 1/2 c), Lm 10 (serotype 1/2 a), Lm 13 (serotype 4b) and Lm 27 (serotype 1/2 a). In addition other members of the *Listeria* genus were used, specifically *L. innocua* 11994 (serotype 6a), *L. ivanovii* NCTC 11846 (serotype not determined) and *L. seeligeri* NCTC 11856 (serotype not determined).

As can be seen in Table 4.4, different cfu: pfu ratios were obtained for the different strains. The results showed that a range of detection efficiencies were achieved for *L. monocytogenes* strains of both serotype 1/2a and 4b (from 1:0.63 to 1:0.02), so that in the worst case 1 in 50 cells were detected by the phage assay. The isolates of *L. ivannovi* and *L. seeligeri* both gave relatively low cfu: pfu ratios (1:0.02 and 1:0.06, respectively). However, surprisingly, *L. innocua* was detected with the lowest efficiency (1:0.001) which may explain why plaques of this phage are relatively small when the phage are grown on *L. innocua*. The results of this investigation suggest that the phage detection assay was able to detect all the species belonging to the genus *Listeria* that were examined and that the detection event was sufficiently sensitive to allow detection of at least 50 cells after the enrichment step.

Table 4.4 The detection of viable different *Listeria* strains using the phage detection assay

Species	Serotype	CFU ml^{-1}	PFU ml^{-1}	CFU: PFU ratio
L. monocytogenes EGD-e	1/2 a	1.2×10^8	1.3×10^7	1:0.11
L. monocytogenes 1	1/2 a	5.5×10^8	8.3×10^6	1:0.02
L. monocytogenes 10	1/2 a	1.6×10^8	3.4×10^6	1:0.02
L. monocytogenes 27	1/2 a	3.8×10^8	8.0×10^7	1:0.23
L. monocytogenes 10403S	1/2 a	4.3×10^8	2.7×10^8	1:0.63
L. monocytogenes 3	1/2 c	4.5×10^8	1.8×10^7	10.04
L. monocytogenes ATCC23074	4b	1.6×10^8	9.6×10^6	1:0.06
L. monocytogenes 13	4b	4.4×10^8	2.2×10^8	1:0.5
L. ivanovii NCTC 11846	-	6.5×10^7	1.5×10^6	1:0.02
L. seeligeri NCTC 11856	-	5.0×10^8	3.2×10^7	1:0.06
L. innocua 11994	6a	5.5×10^8	7.0×10^5	1:0.001

Listeria strains were grown overnight in BHI and ten-fold dilutions prepared in Lambda buffer. Samples (500 µl) of each dilution were transferred into sterile reaction vessel and phage detection assay was done as described in Section 2.5.

4.4 Discussion

The development of rapid and sensitive methods for detection of *L. monocytogenes* in foods has become crucial for responding to greater threats posed by emerging in the recent years during the outbreaks. Phage detection technology was developed for rapid bacterial isolation and identification. This method is based on the interactions between a bacteriophage and its bacterial host coupled with inactivation of un-adsorbed extracellular phage by

phagicidal agent, which can provide rapid detection of pathogens (Stewart *et al*., 1998; Stanley *et al*., 2007). As reported in previous studies, the interactions of bacteriophage and their host bacteria has been successfully applied to various pathogens such as *Pseudomonas aeruginosa*, *Salmonella*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Listeria* and *Campylobacter* (Stewart *et al*., 1998; Rees and Voorhees, 2005; Rees and Dodd, 2006). However Stanley *et al.* (2007) were first to show that the assay could be used to detect pathogens in food samples (raw milk samples). Botsaris *et al*. (2010) showed that the same assay could be successfully applied to cheese. However before a phage assay developed in the laboratory can be successfully exploited, several factors had to be addressed, including the sensitivity and specificity of this assay and its wider applicability.

In this study, the detection of *L. monocytogenes* cells by the phage detection assay was examined. The effectiveness of this method as a practical tool is determined by comparing the cell number of plaques produced on a lawn of non-pathogenic bacteria (*L. innocua*) with the number of colonies produced from original sample. The first indication that the assay was performing in a quantitative manner was when it was noted that the cfu ml^{-1} and pfu ml^{-1} results of a 10-fold *L. monocytogenes* WSLC 1042 culture corresponded, indicating that the number of plaque forming units is correlated to the number of *Listeria* cells in the test sample. However the plaque forming unit (pfu): colony forming unit (cfu) ratio did indicate that some cells in the sample that were detected by viable count were not detected by the phage assay. This is expected, as the

infection process is a probability event related to the binding of the phage to the receptor on the host cell.

The interpretation of the results gained were also seen to be affected by this, as the plaques produced on the lawns of *L. innocua* were of a mixture of sizes, from medium to small (Fig. 4.1). and this meant that some plaques may have not been recorded However, it is known that the formation of plaques when titering phage is influenced by the physical and chemical conditions on the double-agar plates used to grow the host cells, the growth conditions used to incubate the plates and also the efficiency of phage infection for each host strain (Łos *et al*., 2008). From the results in Table 4.4 it is clear that the ability of phage A511 to infect *L. innocua* is not good, but there was no simple way to change this parameter. However to encourage a bigger size of plaque, and to try and improve the correlation between the cfu ml^{-1} and pfu ml^{-1} results, different concentrations of soft agar were tested and this was found to achieve some improvement, presumably by improving plaque growth by increasing diffusion through the agar and allowing cells in a wider area to be infected. Therefore the phage amplification assay procedure was modified to standardly use 0.4 % soft agar.

This assay was being developed to detect *L. monocytogenes* in food samples and these provide very diverse growth conditions and therefore once a model assay system had been developed it was important to determine the effect of culture media on the ability of the assay to detect cells. This assessment was carried out by comparing the assay results with *L. monocytogenes* WSLC 1042 grown in different media such as Fraser broth which is routinely used as a

secondary enrichment broth for the isolation of *Listeria* spp. and contains Esculin to indicate the presence of a potential *Listeria* isolate. According to Guenther *et al*. (2009), the infection of *Listeria* cells by phage A511 is dependent on several parameters, such as the substances which may interfere with this process, and the binding of phage to their ligands on the bacterial surfaces is influenced by the ionic strength of the media. In the assay design, the cells were grown in the broth and then resuspended in Lambda buffer to rule out the possibility that some component of the media was directly preventing the phage binding or infection rather than any change in the ability of the phage to infect the cells being due to a change in the cells surface. Despite the presence of these components in the broth, the results of the phage assay were not affected (the cfu: pfu ratios were similar for BHI and Frazer broth).

Premaratne *et al*. (1991) reported that *L. monocytogenes* is relatively fastidious in nutrient requirements, and it requires sugars, amino acids, vitamins, and a suitable source of iron for growth. The researchers also suggested that *L. monocytogenes* can only use a limited number of sources of carbon for energy, and glucose being the preferred source. Research in this laboratory has recently shown that in addition to being growth limiting, the two defined media, D10 and MCDB 202, affect the physiology of *L. monocytogenes* cells by inducing the production of surface capsular material (Nwaiwu, 2011). Capsule production is known to protect cells against phage infection by physically blocking the binding of the phage to the cell surface. When these media were used the cfu: pfu ratios were lower than when the cells were grown in either

BHI or Fraser broth. This would be consistent with the production of capsules, but could also be because the host cells are not expressing the appropriate receptors on the cells surface for the phage to bind. The cell surfaces of *Listeria* strains are composed of various compounds, which include peptidoglycan, teichoic acids and lipoteichoic acids and the broad-host-range virulent phage A511 (Myovirus) uses the listerial peptidoglycan as primary receptor. It is unlikely that this will be changed when cells are grown in different media but it may be masked by the production of capsular material.

To develop the assay, the propagating host for phage A511 was used. Therefore to investigate whether the assay would be able to detect more members of *Listeria* species e.g., *L. innocua* 11994 (6a), *L. ivanovii* NCTC 11846 and *L. seeligeri* NCTC 11856 and a wider sample of *L. monocytogenes* isolates representing well studied laboratory strains, strains of different serovars and some food isolates were tested. The obtained results from these experiments show that a range of detection efficiencies were achieved, with *L. ivannovi* (1:0.02) and *L. seeligeri* (1:0.06) detected at similar levels. However the lowest ratio was observed with *L. innocua* (1:0.001), which may explain why plaques of this phage are relatively small when grown on *L. innocua*. On the other hand, the results of this study indicated that phage detection assay can detect all the species belonging to the genus *Listeria* tested in this study but that the efficiency of detection was not the same in all cases, so that the sensitivity of the plaque count would be lower than that of the viable count (Table 4.4). This difference is probably related to the ability of the phage to initially bind (reversibly) to the cell surface, and when this binding is less tight

the phage may then fall off the cell which would reduce the efficiency of infection. This finding is in agreement with the reported results by Barbalho *et al*. (2005), in which bacteriophage A511 was used as a screening method for rapid confirmation of *Listeria* species, showing that all suspected colonies, confirmed as *Listeria* through conventional methods, demonstrated their susceptibility to the phage.

Therefore, it can be concluded that a novel phage detection assay for the specific identification of *L. monocytogenes*, *L. innocua*, *L. ivanovii* and *L. seeligeri* has been developed in this study, which could be used for faster detection and avoiding time-delay taken when using classical isolation and could be used as a new approach for direct identification of all the species belonging to the genus *Listeria* rather than using phage to confirm identification after growth as described by Barbalho *et al*. (2005).

CHAPTER 5

DEVELOPMENT OF A COMBINED PHAGE-PCR ASSAY METHOD

5.1 Introduction

Advancements in molecular technology include developments in rapid methods that reduce time, as well as offer great sensitivity and specificity in the detection of pathogens. Molecular epidemiology is based on characterisation of bacterial isolates by using phenotypic assays (e.g. species-specific identification with biochemical standard methods), which include sugar fermentations or the CAMP phenomenon. In the past, several approaches have been described to differentiate *Listeria* strains using biochemical, immunological or bacteriophage lysis techniques to shorten the time required for identification, which most likely would have allowed more rapid monitoring of all these *Listeria* species (Bubert *et al*., 1999). However it still remains a challenge to rapidly and reliably differentiate *L. monocytogenes* isolates from the other *Listeria* species in this genus.

In the field of microbiology molecular, detection techniques are becoming increasingly common as a way of simplifying testing procedures and increasing the specificity of the identification procedure. However these have always proved to be challenging when working with food samples. Several PCR-based strategies have been successfully used for the detection and identification of the presence of *L. monocytogenes* in various sample matrices (Stevens and

Jaykus, 2004; Chen *et al*., 2005). These strategies have offered a shorter detection time, low detection limits, a higher specificity and a potential for automation (Janzten *et al.,* 2006). Although the PCR technique is a powerful technology, and there have been many studies on pathogen detection in foods have used PCR methods (Thomas *et al*., 1991; Croci *et al*., 2004), many problems have been reported associated with the presence of numerous inhibitory compounds in foods and in some selective microbiological media, such as bile salts and acriflavin (Mandal *et al*., 2011). Furthermore, using PCR methods on their own cannot differentiate between viable and dead cells in samples, and hence providing more false-negative results (Flekna *et al.*, 2007).

Since the phage used in this assay must have a broad host range to infect both *L. monocytogenes* and cells of the *Listeria* genus, so that it detects all target cells as well as the helper cells that form the lawn, this introduces issues of specificity. Stanley *et al*. (2007) demonstrated that PCR amplification of genomic signature sequences from the DNA released into the plaque following the phage amplification assay could be used to confirm the identity of the cell detected by the phage. This combination of techniques provides the advantage over standard PCR methods that only viable cells are detected, since the cell must be viable to support phage replication, and the DNA is efficiently extracted from the single cell detected by the phage lysis event improving PCR detection.

When phage infect bacterial cells, many induce enzymes to degrade the host cell genome. Hence it was somewhat surprising that Stanley *et al*. (2007) found that the genomic DNA released from the original cell is preserved long enough

in the plaque to serve as a template for PCR amplification. Therefore there could be no guarantee that this approach would be successful when applied to another phage amplification assay, as it may have been a specific characteristic of the particular mycobacteria phage used in that study.

PCR techniques used to confirm the identity of *L. monocytogenes* isolates have been demonstrated by a number of researchers (for instance Ericsson *et al*., 1997b; Holko *et al*., 2002; Somer and Kashi, 2003; Gouws *et al*., 2005). However, of these, assays described by Somer and Kashi (2003) appeared to be the most suited for this work. The primers involved in this PCR reaction were designed according to the differences in 16S rRNA sequences among *Listeria* spp. and they can differentiate between *L. monocytogenes* and other *Listeria spp.* on the basis of the amplification of two different PCR products. One indicates the presence of *Listeria* spp. and a second band indicates the presence of *L. monocytogenes.* This PCR assay was particularly compatible with the phage amplification assay since plaque samples will always contain *L. innocua* DNA released from the helper cells that form the lawn. Hence when using plaque DNA from the assay as a template, the PCR reaction should always give a positive result. This provides an internal positive control for the PCR typing assay.

The purpose of the work presented in this Chapter was to optimise a molecular multiplex-PCR-based method first described by Somer and Kashi (2003) that was designed to identify all the species of the genus *Listeria* and simultaneously specifically identify *L. monocytogenes* cells, and determine

whether it could be used in combination with the phage amplification assay described in Chapter 4.

5.2 Results

The multiplex PCR described by Somer and Kashi (2003) uses three alternative forward primers designed for the presence of one or more of the *Listeria* spp. The first primer was specific for *Listeria ivanovii* (IVA-F), the second was specific for *Listeria grayi* and *Listeria murrayi* (MG-F), and the third was specific for *L. monocytogenes*, *Listeria innocua, Listeria welshimeri* and *Listeria seeligeri* (LIS-F).

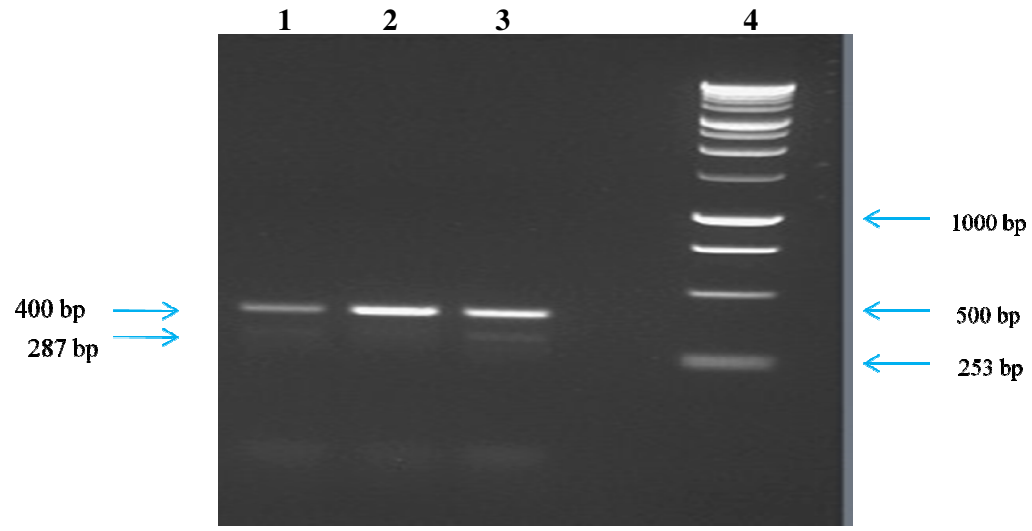
Two forward primers were used for the specific amplification of all *L. monocytogenes* strains, the first primer was (MONO5-F) and the second primer (MONO7-F) is used to increase the coverage because of known variation in the *L. monocytogenes* gene sequence (Somer and Kashi 2003). The reverse primer (LIS-R) was designed for all members of the genus and *L. monocytogenes* (See Table 2.4). Therefore all of the *Listeria* spp. were characterized to amplify a fragment of about 400 bp., whereas *L. monocytogenes* was characterized by two PCR products: one of 400 bp for the genus *Listeria*, and 287 bp for the *L. monocytogenes* strains.

Initially to establish that the PCR assay was working, the multiplex PCR was performed as described by Somer and Kashi (2003) in a 25 µl reaction mixture

containing 1.5 mM $MgCl_2$, 5 mM dNTPs, 10 μM of each of the primers with 10 μl of template DNA purified by a simple heat lysis method (Section 2.10.1). At the same time the ability for this assay to amplify *L. monocytogenes* from DNA extracted from a plaque was tested. This DNA was purified by using the method described by Stanley *et al*. (2007) (Section 2.10.2). The published PCR method conditions used 5 cycles with an annealing temperature of 63 °C for 45 s, and then 20 cycles with an annealing temperature of 58 °C for 45 s.

The results (Fig. 5.1) showed that using both the plaque DNA and the genomic DNA, the *Listeria* spp. band of 400 bp was successfully amplified. However using the genomic DNA the *L. monocytogenes*-specific bands were only weakly amplified, and this band was not detected using the plaque DNA. Since the plaque DNA contains only a low concentration of *L. monocytogenes* template DNA and an excess of DNA from the lysed *L. innocua* cells that form the lawn, if it was not possible to amplify the *L. monocytogenes*-specific band using DNA extracted only from these cells it is not surprising that the plaque PCR amplification was not successful. In addition to the problem with a low concentration of template DNA, it is also possible that components of the media present in the plaque DNA sample could also interfere with the PCR amplification.

Figure 5.1 Result of multiplex PCR using both genomic and single plaque DNA templates



Gel showing PCR products generated using the multiplex PCR conditions described by Somer and Kashi (2003). Template DNA used was lane 1, 10 µl plaque DNA; lanes 2 and 3, 10 µl *L. monocytogenes* WSCL1042 genomic DNA (replicate experiments); lane 4, 1 kbp marker (Promega). Sizes of key marker fragments are indicated. The PCR conditions used were 1 cycle at 95 °C for 5 min; 5 cycles at 95 °C for 45 s, at 63 °C for 45 s, at 72 °C for 45 s; 20 cycles at 95 °C for 45 s, at 58 °C for 45 s, at 72 °C for 45 s and 1 cycle at 72 °C for 7 min. The expected sizes of the products were 400 bp for all *Listeria* spp. and 287 bp for *L. monocytogenes*. PCR products were examined on a 2 % TAE agarose gel at 80 V for 2 h.

5.2.1 Preparation of Genomic DNA

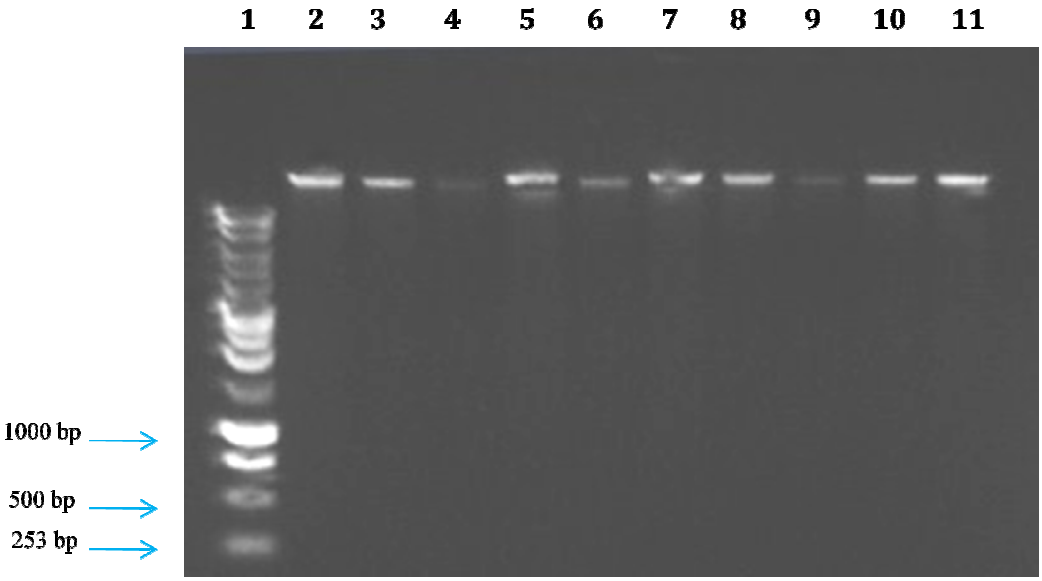
To try and improve the results obtained from PCR, good quality DNA was needed. The simple heat lysis method describe by Stanley *et al.* (2007) does not remove any cell debris from the sample and therefore this method was replaced with a commercial DNA extraction kit. Therefore a GenElute™ bacterial genomic DNA kit was used to purify DNA from colonies of different

strains of *L. monocytogenes* (Section 2.10.3) and the DNA extracted was visualised on an agarose gel (Figure 5.2, lanes 2-6).

In addition a modification of the GenElute^{TM} method was used to purify DNA from individual plaques produced from the phage amplification assay (Section 2.10.3). In this case the plaques were isolated from the phage amplification assay that had been used to detect the same strains of *L. monocytogenes* as above. A plaque was resuspended in 10 µl of SDW and the agar was then melted at 95 °C for 5 min. This was kept molten by cooling to 37 °C and then the kit used to extract DNA directly from the samples. It should be noted that this plaque DNA consists of a low quantity of *L. monocytogenes* genomic DNA (theoretically one genome coming from the original cell detected by the assay) and an excess of *L. innocua* DNA coming from the lysed cells that form the lawn, and this DNA acts as a carrier to help recover the low amount of *L. monocytogenes* genomic DNA present in the plaque.

The first step of the kit procedure is the addition of 200 µl of a lysozyme/lysis solution and incubating the sample at 37 °C for 30 min. Although not necessary as the cells in the plaque were already lysed, this step was performed since the addition of this volume of liquid diluted the agar in the sample and ensured that the plaque sample did not solidify again. The plaque DNA extracted using this method is shown in Figure 5.2, (lanes 7-11) and the results obtained indicated that the amount of genomic DNA purified from each of the samples was of high quality, as no obvious smearing/degradation of DNA was observed and was of sufficient quantity for downstream applications.

Figure 5.2 **Purification of *L. monocytogenes* genomic DNA and single plaque DNA using the GenElute™ bacterial genomic DNA kit**



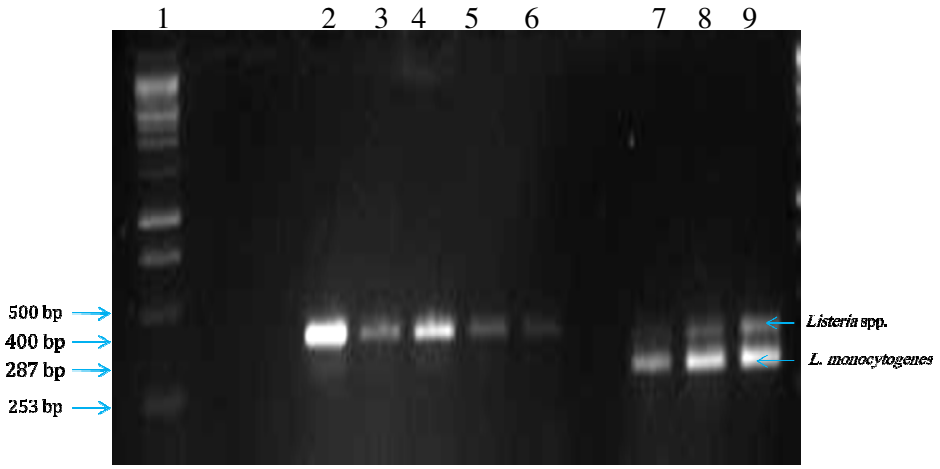
The DNA was extracted using the GenElute™ bacterial genomic DNA kit and was analysed on a 0.8 % TAE agarose gel at 70 V for 1h. Lane 1, 1 kbp molecular size marker. Lane 2-6 chromosomal DNA extracted from colony of *L. monocytogenes* and (lane 7-11) from plaque DNA resulting from the phage amplification assay. Strains of *L. monocytogenes* used in each case were lanes 2 and 7, *L. monocytogenes* WSLC 1042, lanes 3 and 8, EGD-e; lanes 4 and 9, 23074; lanes 5 and 10, *L. monocytogenes* 1, lanes 6 and 11; *L. monocytogenes* 3.

5.2.2 Optimisation of the PCR Method

Once a DNA extraction method had been established, the multiplex PCR reaction was optimized by changing the parameters described by Somer and Kashi (2003). Initially the amplification efficiency at different annealing temperatures (e.g. 53 °C to 63 °C) was tested using the genomic DNA extracted from *L. monocytogenes* WSCL 1042 cells using the GenElute™

method (Section 2.10.3). In this experiment, the best results were obtained using an annealing temperature of 53 °C which now allowed the amplification of the *L. monocytogenes* specific band in addition to the *Listeria* spp. band and the standard PCR conditions chosen for future work were 1 cycle at 95 °C for 5 min; 5 cycles at 95 °C for 45 s, at 53 °C for 45 s, at 72 °C for 45 s; 20 cycles at 95 °C for 45 s, at 58 °C for 45 s, at 72 °C for 45 s; and 1 cycle at 72 °C for 7 min. Although two bands were amplified at this lower annealing temperature, the intensity of the upper band (*Listeria* spp.) was now reduced compared to the intensity of the lower band (Fig. 5.3).

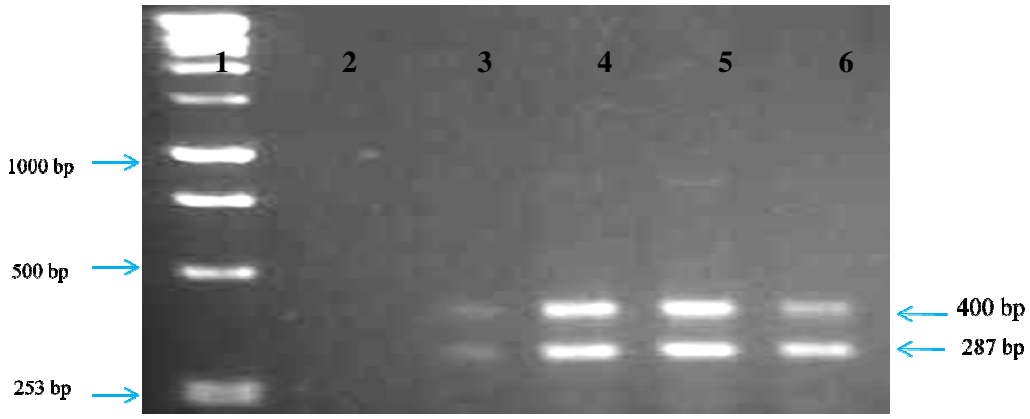
Figure 5.3 Optimising PCR annealing temperature



PCR amplification using genomic DNA of *L. monocytogenes* WSLC 1042 and plaque DNA after using *L. monocytogenes* WSLC 1042 as the target for the phage amplification assay. Lane 1; molecular weight marker (Promega 1 kbp ladder), lane 2-6; plaque DNA, lane 7, 8, 9; genomic DNA of *L. monocytogenes* WSCL 1042. PCR products were examined on a 2 % TAE agarose gel at 80 V for 2 h

To try and improve the amplification efficiency of the bands, the magnesium chloride ($MgCl_2$) concentration was increased from 1.5 mM to 1.7 mM and the amount of LIS-R primer (the Universal reverse primer needed for all the PCR reactions) was increased from 1.5 µl to 1.7 µl. These changes resulted in increased amplification of the additional band (*L. monocytogenes*) using the optimised standard PCR conditions (Fig. 5.4, lane 6).

Figure 5.4 Results of Optimised PCR assay and investigation of sensitivity of multiplex PCR reaction.



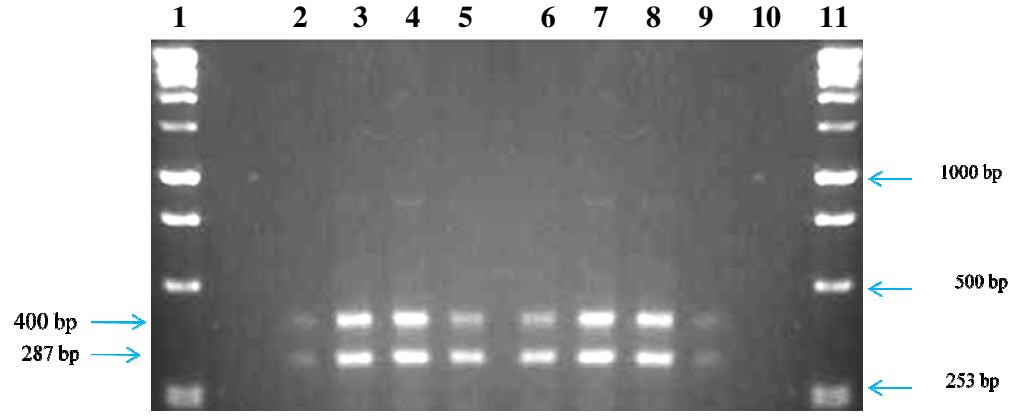
Results of multiplex PCR for identifying *L. monocytogenes*. Samples were analysed on a 2 % TAE agarose gel, run for 1.5 h at 80 V. Template DNA was extracted from *L. monocytogenes* WSCL 1042 cells using the GenElute™ method. Lane 1: The molecular weight marker (Promega 1 kbp ladder), lane 2; 6.0 µl template DNA, lane 3; 7.0 µl template DNA , Lane 4; 8.0 µl template DNA , lane 5; 9.0 µl template DNA and Lane 6, 10 µl template DNA. 400bp band is *Listeria* spp.; 287bp band is *L. monocytogenes*.

Since this experiment was performed using just *L. monocytogenes* DNA, but the plaque DNA would contain more limited amounts of *L. monocytogenes* DNA, an experiment was performed to see if the PCR reaction still worked using smaller amounts of template DNA. To do this the amount of template DNA added was decreased from 10 µl to 6 µl in 1 µl increments. When this was done it was found that a minimum of 8 µl of template DNA was needed to achieve good amplification. Therefore when applying this to plaque DNA samples it would be important to determine if sufficient template DNA could be recovered from the plaques to meet this limitation.

5.2.3 Specificity of the Multiplex PCR

To demonstrate that the optimised PCR conditions worked equally well with all isolates of *L. monocytogenes,* the multiplex PCR assay was further evaluated using different reference strains and a range of food isolates. Specifically the strains used were *L. monocytogenes* 1 (serotype 1/2a), *L. monocytogenes* 3 (serotype 1/2 c), *L. monocytogenes* 10 (serotype 1/2 a), *L. monocytogenes* 4 (serotype 1/2 a), *L. monocytogenes* EGD-e (serotype 1/2 b), *L. monocytogenes* ATCC 23074 (serotype 4b), *L. monocytogenes* 27 (serotype 1/2 a), *L. monocytogenes* 10 (serotype 1/2 a), *L. monocytogenes*10403S (serotype 1/2 a). DNA extracted from *Salmonella* spp. was used as a negative control rather than adding no template at all (this DNA was used as it was available in the laboratory and should not produce a PCR product with the primers). Figure 5.5 shows the multiplex PCR results obtained using 10 µl of genomic DNA extracted using the GenElute^{TM} method from the different *L. monocytogenes* stains. The primers formed expected dual bands of 400 bp and 287 bp for all the *L. monocytogenes* strains tested (Fig. 5.5 lanes 2- 9) and no DNA product was seen for the *Salmonella* spp. genomic DNA used as a negative control sample.

Figure 5.5 Testing multiplex PCR with different *L. monocytogenes* strains

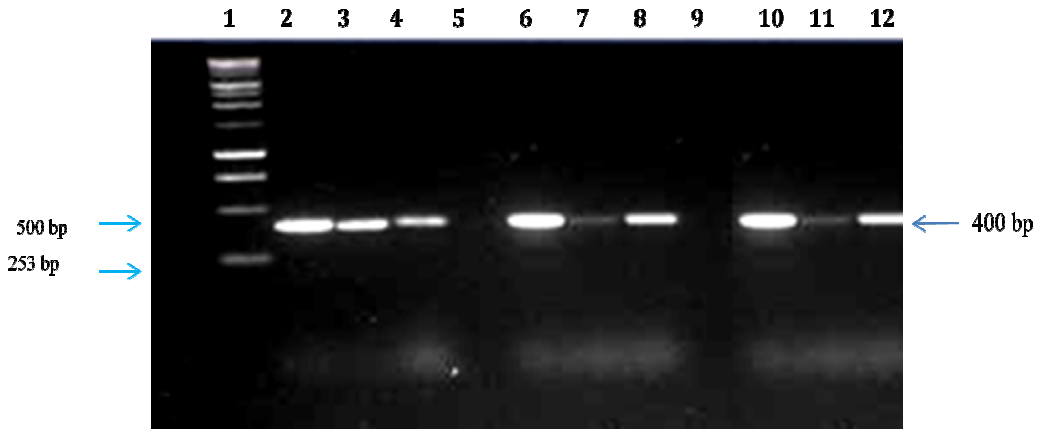


PCR amplification using genomic DNA of different *L. monocytogenes* strains. Lane 1 and Lane 11: The molecular weight marker (Promega 1 kbp ladder). Lane 2; *L. monocytogenes* 1 , lane 3; *L. monocytogenes* 3 , lane 4; *L. monocytogenes* 10, lane 5; *L. monocytogenes* 4, lane 6; *L. monocytogenes* EGD-e, Lane 7; *L. monocytogenes* ATCC 23074, lane 8; *L. monocytogenes* 27, lane 9; *L. monocytogenes* 10403S and lane 10; *Salmonella* spp. (negative control). Samples were analysed on a 2 % TAE agarose gel and PCR products were separated for 1.5 h at 70 V. 400bp band is *Listeria* spp. and 287 bp *L. monocytogenes*.

The multiplex PCR contains forward primers specific for *L. ivanovii* (IVA-F), *L. grayi* and *L. murrayi* (MG-F), and all other members of *Listeria* genus (LIS-F). All these primers should produce a 400 bp product. Figure 5.6 shows the results gained when the multiplex PCR was performed using genomic DNA extracted from *L. innocua*, *L. seeligeri* and *L. ivanovii* strains using three independent replicate PCR amplifications. In all cases the expected product size of 400 bp was obtained without any non-specific DNA amplification of the *L. monocytogenes*-specific band and no detectable product was produced in the

negative control samples. However it was noted that the band intensity of the *Listeria* spp. 400 bp product was variable.

Figure 5.6: Testing multiplex PCR with different *Listeria* spp.



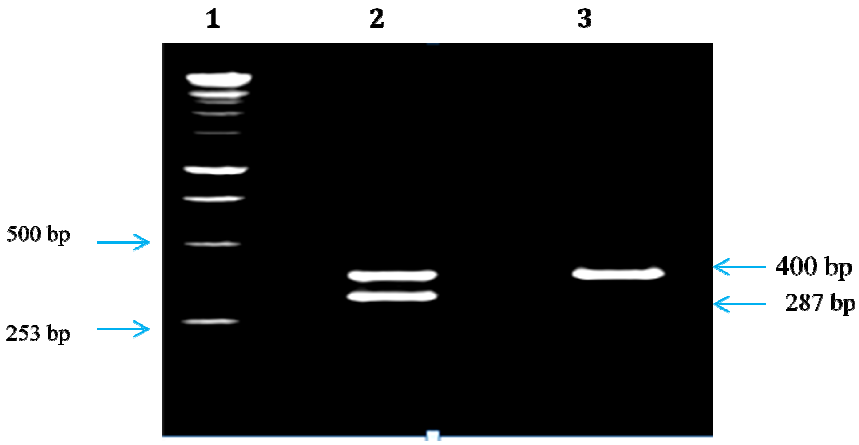
PCR amplification using genomic DNA of different *Listeria strains*. Lane 1; molecular weight marker (Promega 1 kbp ladder), lane 2; *L. innocua* 11994 (replicate experiments), lane 5; No DNA control, lane 6-8; *L. ivanovii* NCTC 11846 (replicate experiments), lane 9; *Salmonella* spp. DNA (negative control), lane 10-12; *L. seeligeri* NCTC 11856 (replicate experiments). Samples were analysed on a 2 % TAE agarose gel and PCR products were separated for 1.5 h at 80 V. 400bp band is *Listeria* spp.

5.2.4 PCR-Amplification of Plaque DNA

Now that the multiplex PCR had been evaluated using genomic DNA, the method was tested to see if it was compatible with the phage amplification assay. Initially DNA was extracted from the centre area of a plaque from phage amplification detection assay by cutting off a tip (approximately 2 mm) to pick up the plaque. Following the DNA extraction using the GenElute^{TM} Bacterial Genomic DNA Kit (Section 2.10.3), 10 µl of the DNA was used for the multiplex PCR performed in a 25 µl reaction mixture (Section 2.10.4).

As shown in Figure 5.7 two bands of 287 bp and 400 bp were produced from plaque DNA when *L. monocytogenes* WSLC was used as the target for the phage amplification assay. This result indicated that the components of the agar did not inhibit the PCR and the concentration of the DNA extracted was sufficient to allow good amplification of both PCR products, even when the *L. monocytogenes* DNA sample contained an excess of *L. innocua* DNA.

Figure 5.7 Results of multiplex PCR using DNA extracted from single plaques

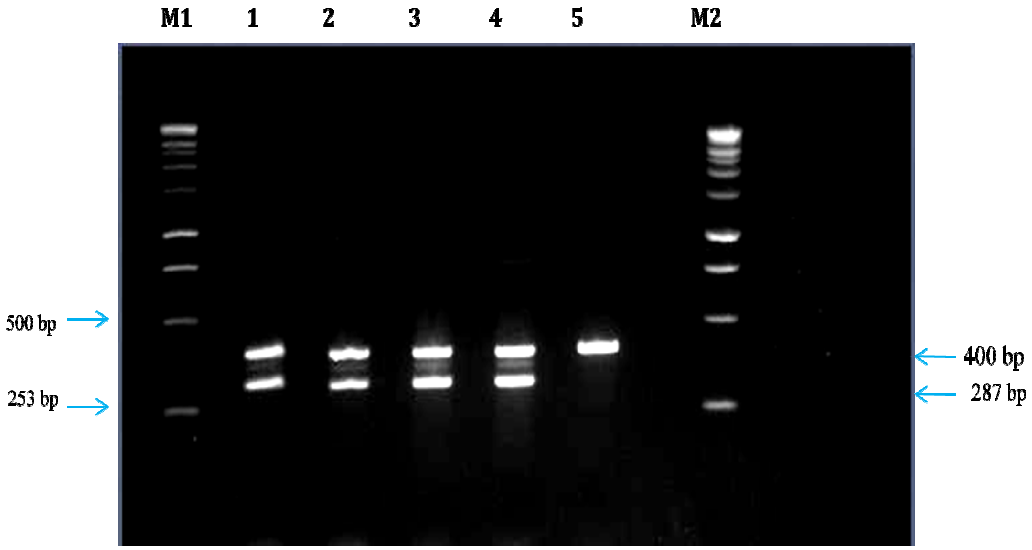


PCR amplification of products from purified plaque DNA. Lane 1: molecular weight marker (Promega 1 kbp ladder), Lane 2; plaque DNA after using *L. monocytogenes* WSLC 1042 as the target for the phage amplification assay, lane 3; plaque DNA after using *L. innocua* 11994 as the target for the phage amplification assay. PCR products were examined on a 2 % TAE agarose gel at 80 V for 2 h (Section 2.10.5).

In contrast when plaque DNA was prepared when *L. innocua* or *L. ivanovii* was used as the target for the phage amplification assay only one PCR band of 400 bp was produced (Figures 5.7 and 5.8). Hence from these experiments it was shown that the multiplex PCR assay was able to distinguish between *L.*

monocytogenes and all other *Listeria* species after extraction of template DNA from the plaques.

Figure 5.8 Results of multiplex PCR using DNA extracted from single plaques

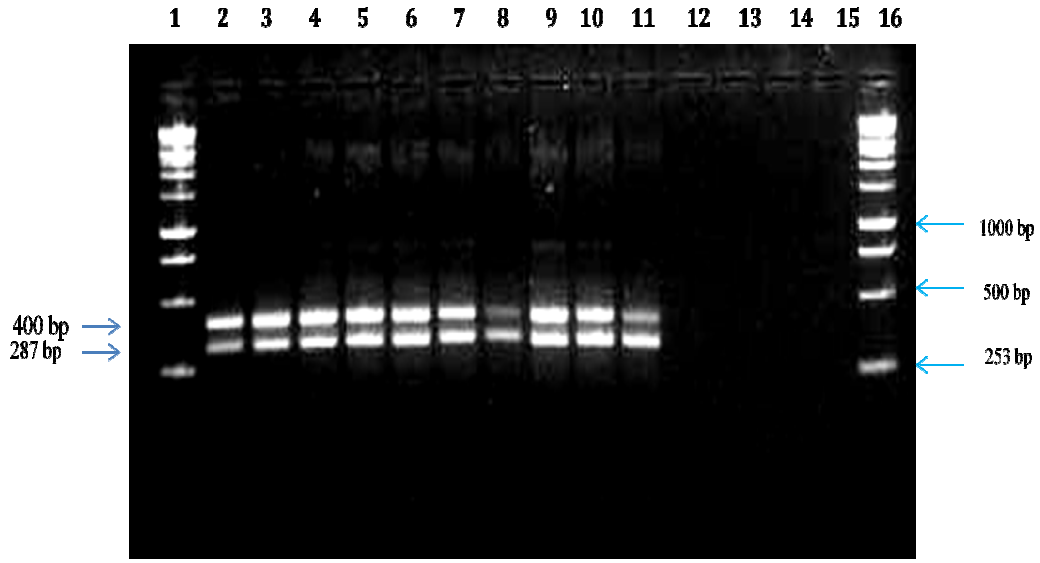


PCR amplification of products from purified plaque DNA. Lanes M1 and M2: molecular weight marker (Promega 1 kbp ladder), lane 1-4; plaque DNA after using *L. monocytogenes* WSLC 1042 as the target for the phage amplification assay (replicate experiments), lane 5; plaque DNA after using *L. ivanovii* 11846 as the target for the phage amplification assay. PCR products were examined on a 2 % TAE agarose gel at 80 V for 2 h (Section 2.10.5).

This experiment was extended to investigate the sensitivity of the multiplex PCR when using plaque DNA as the PCR template. In this experiment the amount of plaque DNA added to the PCR reaction was reduced from 10 µl to 4 µl in 1 µl steps to determine the lowest concentration that could be used and successfully achieve detection of the target DNA. The PCR amplification was

carried out using the optimised PCR conditions described earlier in this Chapter. As shown in Figure 5.9, the 400 bp and 287 bp bands, which lead to the identification of *L. monocytogenes,* were successfully amplified in this experiment until only 50 % of the normal amount of template DNA was added to the PCR mixture. This result indicated that the amount of DNA that was being extracted from a single plaque was in excess and that even if the DNA extraction was only 40 % efficient, the reaction was sensitive enough to detect the genomic DNA of the original cell detected in the phage assay.

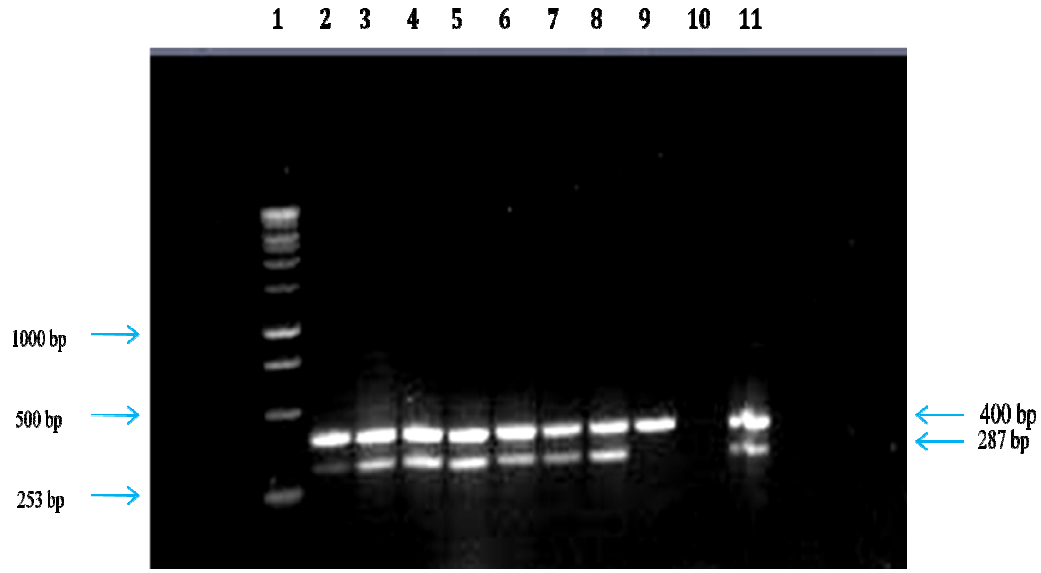
Figure 5.9 Demonstrating limit of detection of multiplex PCR for detection of *L. monocytogenes*



PCR amplification of products from different volumes of purified plaque DNA. Lanes 1 and 16; molecular weight marker (Promega 1 kbp ladder).Lane 2-15 shows the results obtained using decreasing amounts of plaque DNA after using *L. monocytogenes* WSLC 1042 as the target for the phage amplification assay template for the PCR assay. Each test was performed in duplicate. Lanes 2 and 3; 10 µl template DNA, lanes 4 and 5; 9.0 µl template DNA, lanes 6 and 7; 8.0 µl template DNA, lanes 8 and 9; 7.0 µl template DNA, lanes 10 and 11; 6.0 µl template DNA, lanes12 and 13; 5.0 µl template DNA and lanes 14 and 15; 4.0 µl template DNA. PCR products were examined on a 2 % TAE agarose gel at 80 V for 1.5 h (Section 2.10.5).

To confirm that the PCR reaction was able to detect a wide range of *L. monocytogenes* cell types following the phage amplification assay, a range of different strains was tested which included a number of isolates of different serotypes that had been isolated from foods (*L. monocytogenes* 1 (serotype 1/2a), *L. monocytogenes* 3 (serotype 1/2c), *L. monocytogenes* 10 (serotype 1/2 a), *L. monocytogenes* 4 (serotype 1/2a) and *L. monocytogenes* 27 (serotype 1/2a) and some standard laboratory strains (*L. monocytogenes* EGD-e (serotype 1/2a) and *L. monocytogenes* ATCC23074 (serotype 4b). For each of these strains, the phage amplification assay was performed and DNA extracted from the resulting plaques. In addition plaque DNA resulting from the detection of *L. innocua* 11994 was used as a control. It can also be seen that the PCR procedure was able to detect *Listeria* spp. DNA (400 bp band) in all strains tested, including *L. innocua* (Figure 5.10) and for all samples where *L. monocytogenes* species was used in the phage detection assay, two PCR products were evident confirming that *L. monocytogenes* DNA was detected.

Figure 5.10 Use of multiplex PCR to identify different *L. monocytogenes* isolates



PCR amplification of products from purified plaque DNA. Lanes 1; molecular weight marker (Promega 1 kbp ladder), lane 2-8 shows the results obtained using plaque DNA when different *L. monocytogenes* strains were used as the target for the phage amplification assay. Lane 2; *L. monocytogenes* 1, lane 3; *L. monocytogenes* 3, lane 4; *L. monocytogenes* 10, lane 5; *L. monocytogenes* 4, lane 6; *L. monocytogenes* EGD-e, lane 7; *L. monocytogenes* ATCC 23074, lane 8; *L. monocytogenes* 27. Lane 9; plaque DNA when *L. innocua* 11994 was used as the target for the phage amplification assay. Lane 10: Blank (No sample added). Lane 11: template used was purified genomic DNA from *L. monocytogenes* WSCL 1042 (positive control). PCR products were examined on a 2 % TAE agarose gel at 80 V for 2 h (Section 2.10.5).

5.3 Discussion

Because of the close morphological and biochemical similarities of the different members of the *Listeria* genus, and in view of the large number of different techniques and the diverse procedures available for their detection and differentiation (Liu *et al.*, 2004), any new methodology for detection and isolation of *L. monocytogenes* has to be superior in many aspects. These parameters include rapidity, sensitivity, specificity, cost and ease of use. Molecular identification using PCR has already been shown to be capable of easily distinguishing *L. monocytogenes* from other *Listeria* species and has been found to be intrinsically more accurate and less affected by natural variation than the phenotypic methods (Liu, 2006). This is particularly true since typical *L. monocytogenes* isolates have been found that are more difficult to isolate when conventional culture-based methods are used (Leclercq, 2004; Stessl *et al.,* 2009). However there are also problems associated with direct detection of *L. monocytogenes* in food, for example the PCR assays alone cannot give live/dead differentiations (Flekna *et al.*, 2007).

Given this background, the optimisation of methods for detection of *L. monocytogenes* is very necessary, especially when developing a PCR-based assay. Multiplex PCR for molecular detection assays which amplifys more than two amplicons in a single PCR reaction are favoured as they allow the inclusion of internal positive controls and have been shown to be very useful for identification and characterisation of bacterial isolates (Jeyaletchumi *et al*., 2010).

The multiplex PCR reaction used in this study was developed by Somer and Kashi (2003) and the primers were designed to detect differences in the 16S rRNA sequence of *Listeria* spp. This is a good target for detection when an organism is known to show phenotypic variation since it targets conserved DNA regions in the bacterial genome that are less likely to vary. In this case the PCR product found to be universally amplified from all members of the genus was 400 bp whereas the *L. monocytogenes*-specific product from the same region of DNA was 287 bp. The difference in size in the two amplicons meant that they were easily differentiated on a standard agarose gel which makes the assay simple to perform and interpret.

When the PCR assay was attempted using the conditions published by Somer and Kashi (2003), it was found that the assay was not very reproducible. Each laboratory tends to use different reagents, different enzymes, different volumes for the PCR reactions and PCR blocks with different heating/cooling profiles when carrying out PCR. Since all these factors can affect PCR amplification it is common to find that published PCR methods need optimising before they will work efficiently in a new laboratory. In this case some of the concentrations of the PCR reaction components and the primary annealing temperature were adjusted to improve the amplification. When 1.7 mM $MgCl_2$ was used and the concentration of the universal LIS-R primer was increased, the amplification was improved and both expected PCR products became clearly visible. The alteration of the $MgCl_2$ concentration relates to the fact that it is an essential cofactor for the DNA polymerase and also promotes primer binding during the annealing step, so it is important that this is considered

when primer concentration is increased. In addition it was found that preparing the template DNA prepared using a GenElute™ bacterial genomic DNA kit improved the reproducibility of the PCR detection reactions, and therefore this was used rather than using in-house extraction methods that were more liable to variation. Therefore optimization of those components for simultaneous amplification of multiple targets is generally found to be beneficial (Markoulatos *et al*., 2002).

The optimised PCR assay was tested using a range of different *Listeria* species and *L. monocytogenes* isolates, and in accordance with the findings of Somer and Kashi (2003) it was found to be able to amplify DNA from all members of the genus tested and was also able to identify all *L. monocytogenes* isolates by amplification of the species-specific additional band. However the most important finding in this Chapter was that the approach that Stanley *et al*. (2007) described to add specificity to the phage amplification assay by extracting DNA from plaques at the end of the detection assay, and using PCR amplification of signature sequences to identify the cell detected by the phage, was also possible using *Listeria* phage. As previously stated, it is known that many phage induce degradation of host DNA during infection and therefore it was possible that the target cell DNA would not be detectable at the end of the phage amplification assay. In this study it was found that using the GenElute™ bacterial genomic DNA kit improved the efficiency of the PCR reaction, and that using this kit the amount of plaque DNA required as a template was only 6 µl meaning that the plaque DNA extraction step did not have to be 100 % efficient to ensure that the DNA could be detected.

The combined phage-PCR assay was found to be able to detect and identify strains representing various different serotypes of *L. monocytogenes* (serotypes 1/2a, 1/2c and 4b) which represent the major serotypes associated with food-poisoning and was equally effective using well characterised laboratory strains or more recent food isolates. In addition the assay could detect and differentiate between *L. monocytogenes* and *L. innocua* and *L. ivanovii* which are two strains that can be found in food samples. These findings suggest that the assay developed is relatively robust and was suitable for testing with real food samples.

CHAPTER 6

OPTIMISATION OF PHAGE ASSAY FOR DETECTION OF *LISTERIA MONOCYTOGENES* IN STILTON CHEESE

6.1 Introduction

The food borne pathogen *L. monocytogenes* has been a common contaminant in various food factory environments. Food products, especially cheeses and other milk based products have been recognised as an important transmission route of human listeriosis. The incidence of *L. monocytogenes* in soft and semi-soft cheese was found to vary from 0.5 % to 46 % and this resulted in immediate concern in the dairy industry (Abrahão *et al*., 2008). Several outbreaks of listeriosis world wide have been associated with the consumption of soft cheese. For instance in 2002 in Canada an outbreak of 17 cases was associated with soft and semi-hard cheese that had been improperly pasteurised (Gaulin *et al*., 2003). In Sweden in 2001, 48 cases of listeriosis were associated with raw milk cow, goat and blended milk fresh cheeses (Carrique-Mas *et al*., 2003). However the largest reported outbreak associated with soft chees occurred between 1983-1987 in Switzerland when there were 122 cases reported cases linked to Vacherin Mont D'Or soft cheese (Büla *et al*., 1995). Hence *L. monocytogenes* is often associated with causing illness in consumers from consumption of soft cheeses.

Soft cheese is made using different techniques. A generalised procedure for these entails warming pasteurised milk and adding a starter culture to assist

with coagulation by lowering the pH prior to addition of rennet. The temperatures, times, pH adjustments at different steps, whether or not salting is used and how the blocks of cheese are formed vary considerably among different cheese kinds. Typical starter bacteria include *Lactococcus lactis* subsp. *Lactis, Lactobacillus helveticus* and *Lactobacillus delbruckii* subsp (Ardöa and Petterssona, 1988; Ercolini *et al*, 2003) and these are also very variable. Therefore *L. monocytogenes* can enter cheese at different stages through different routes during production. *L. monocytogenes* is also found in raw milk, being excreted by infected animals. Therefore *Listeria* existing in raw milk are able to potentially make their way into the cheese processing plants (Cotton and White, 1992). The environmental diversity of dairy processing plants offers the bacteria a wide range of different locations for colonisation.

In the UK and Europe, there has been an increase in the incidences in the number of reported cases of Listeriosis during the past decades (Goulet *et al*., 2008; Mook *et al*., 2010). Hence development of efficient and rapid methods for detection of this microorganism in foods has been of great significance and is needed to ensure the safety of consuming these foods that are considered to be of higher contamination risk. The conventional bacteriological methods for the detection and quantification of *L. monocytogenes* (ISO 11290-1/A1-1996) are time consuming. In the USA producers must show absence of *L. monocytogenes* from 25 g samples for foods that can support growth of the organism (Martín *et al.*, 2004). Therefore, the development of a quicker and more reliable test, capable of detecting very low numbers of the organism in

ready-to-eat products has also been of high significance to the food industry. To this end the efficacy of the new phage amplification assay was tested using cheese (Stilton) samples. This is a British, semisoft, blue cheese obtained from pasteurised cows' milk (Ercolini *et al*., 2003). These types of cheese products often provide excellent conditions for bacterial growth, which is mainly due to the rise in pH that occurs during ripening (Hicks and Lund, 1991).

6.2 *Listeria monocytogenes* Identification in Cheese

When using traditional cultures to detect *Listeria* in cheese products, the standard ISO 11290 method requires enrichment and incubation for at least 5 days. The purpose of this study was to determine the feasibility of the new phage assay to detect *Listeria* in enrichment samples during the incubation period in order to substantially shorten the detection time required. The target was to reduce the time needed for the confirmation of *Listeria* species to approximately two days instead of five.

6.2.1 Procedures For The Isolation of *Listeria monocytogenes*

Stilton cheese was used in this study and samples of the cheese homogenates were inoculated with *L. monocytogenes* WSLC 1042 using the four procedures described below. A summary of the overall experimental design is given in Figure 6.1.

Procedure I

Slices of 25g of cheese were packed in a polyethylene package and stored at 4 °C until use. Afterwards, the prepared slices were contaminated by spiking them with 0.01, 0.1, 0.5 and 1.0 ml of suspensions of *L. monocytogenes* WSLC

1042 cells (Section 2.3.2) that had been diluted in MRD corresponding to approximately 2, 22, 135 and 2.5 × 10^5 CFU ml^{-1}, respectively (see Fig. 6.1). All samples were prepared in duplicate and they were then homogenised in a laboratory homogeniser (Stomacher 400, Seward) at 230 rpm for 2 min with 225 ml of half-Fraser broth (Section 2.1.11). All samples were incubated at 30 °C for 24 h as a primary enrichment step, before aliquots (0.1 ml) of these samples were then aseptically added to 10 ml of Fraser broth for a secondary enrichment step and incubated at 37 °C for 4 days. Finally 0.1 ml aliquots of these samples were withdrawn for analysis with the phage detection assay (Section 2.5). Parallel samples were direct diluted on Oxford agar plates and incubated at 37 ^oC for 24 h. Further physiological examination of the suspect *Listeria* colonies were performed on the samples as confirmatory tests. These tests included Gram stain, ß-hemolysis on Blood agar plates, catalase reaction, oxidase test and motility at room temperature.

Procedure II

Aliquots of 0.1 ml of *L. monocytogenes* were spiked into slices of 25g of Stilton cheese, and were added to 225 ml of half Fraser broth, homogenised by a laboratory homogeniser (Stomacher 400, Seward) at 230 rpm for 2 min. The enrichment samples were incubated at 30 °C for 22 h. Then portions of 80 ml of these samples were centrifuged at 885 ×*g* for 10 min., the pellet resuspended in 20 ml of Fraser broth and then incubated at 37 °C with continuous shaking for up to 24 h. Portions of 1 ml from each sample were then centrifuged at 10960 ×*g* for 5 min, and the pellet resuspended in 0.1 ml Lambda buffer.The phage amplification assay was then used to detect *L. monocytogenes* in the

prepared samples as described earlier (Section 2.5). Samples from Fraser broth were also streaked on Oxford agar plates and incubated at 37 ^{o}C for 24 h. and afterwards, five suspected *Listeria* colonies were picked from Oxford agar plates for identification and confirmation by further conducting Gram staining, Catalase test, oxidase test, β-haemolysis and hydrolysis of esculin tests.

Procedure III

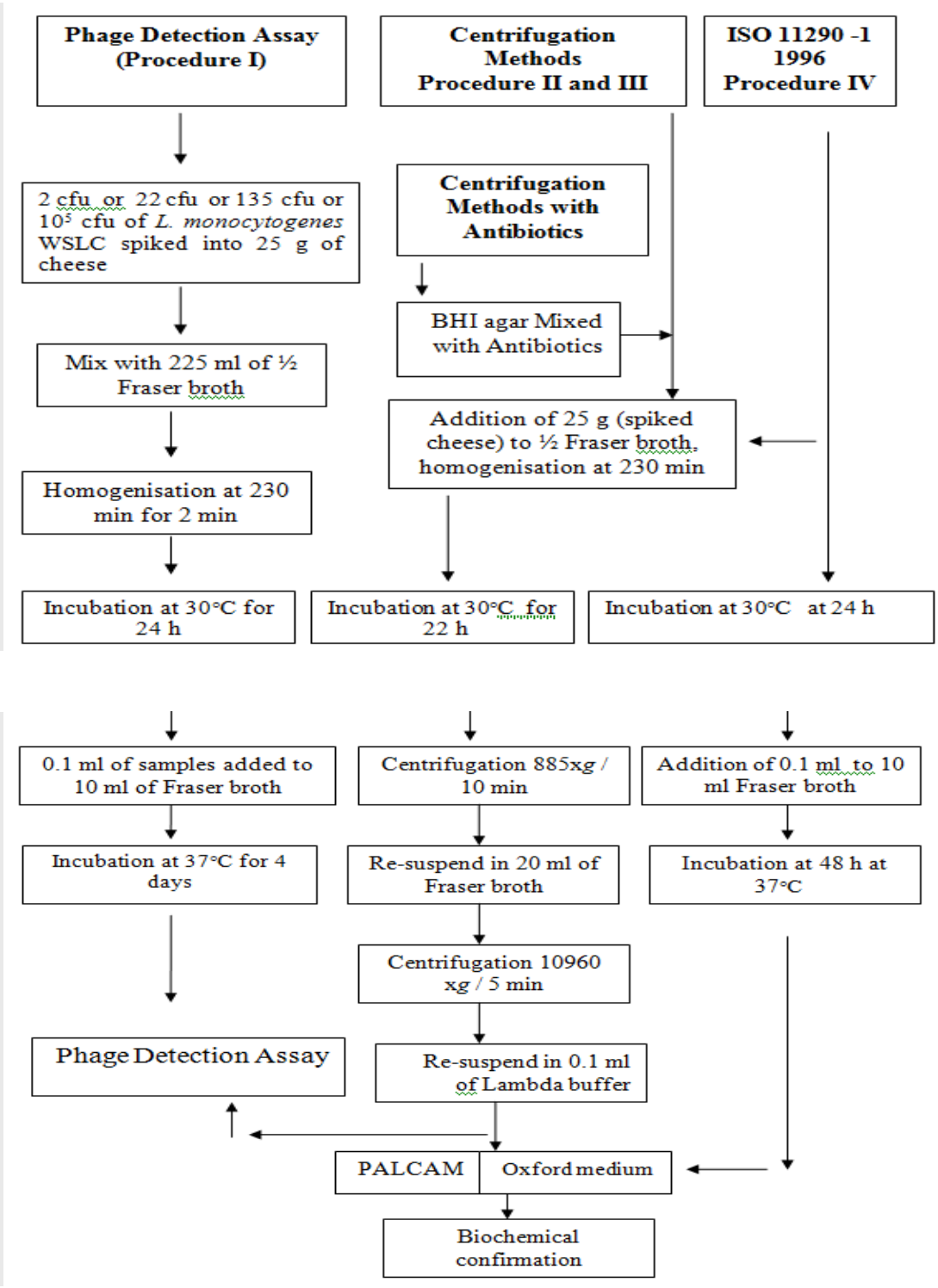
This experiment was similar to Procedure III with the exception of the addition of antibiotics to the Brain Heart Infusion agar used for the phage amplification assay. NAO antimicrobial supplement (Aztreonam 30 µg ml^{-1}, Oxacillin 2 µg ml^{-1} and Nystatin 50 IU ml^{-1} final concentration; Biotec laboratories) was prepared and used according to the manufacturer's instructions. To prepare in house CF antibiotic supplement (0.4 mg ml^{-1} Cycloheximide and 0.01 mg ml^{-1} Fosfomycin final concentration), 40 mg ml^{-1} Cycloheximide, 1 mg ml^{-1} Fosfomycin solution in 70 % ethanol was prepared. This was stored in 1 ml aliquots at -20 ºC until used.

6.2.2 Conventional Culture Method (ISO 11290-1) (Procedure IV)

The detection of *L. monocytogenes* WSLC was conducted according to ISO 11290-1-1996 method. Aliquots of 1.0 ml (10^5 cfu) of *L. monocytogenes* were spiked into 25 g of cheese, and were added to 225 ml of half Fraser broth, homogenised by a laboratory homogenser (stomacher 400, England) at 230 rpm, for 2 min. The samples were then incubated at 30 ^{o}C for 24 h. Portions of 0.1 ml of the incubated samples were added to 10 ml of Fraser broth, and then further incubated for 48 h at 37 ^{o}C. 0.1 ml portions of these samples were

withdrawn and plated on both Oxford agar plates and Polymyxin Acriflavine Lithium Chloride Ceftazidime Aesculin Mannitol (PALCAM) and incubated at 37 ^{o}C for 24 h. If bacterial growth was detected, the suspected *Listeria* colonies (e.g. characteristic black or dark green colonies surrounded by a black‟halo‟) were considered to be presumptive *Listeria* spp. Five of these suspected colonies were picked up and purified by streaking on Tryptone Soya Agar,the plates incubated at 37 °C for 24 h. Biochemical and physiological examinations of the *Listeria* cultures were conducted, and further confirmatory tests including Gram stain reaction, ß-hemolysis on blood agar plates, catalase reaction, oxidase test and motility at room temperature were performed.

Figure 6.1: Flowchart of protocols used for detection of *L. monocytogenes* in Stilton cheese.



6.3 Results

6.3.1 Detection of *L. monocytogenes* in Contaminated Cheese Using the Phage Amplification Assay

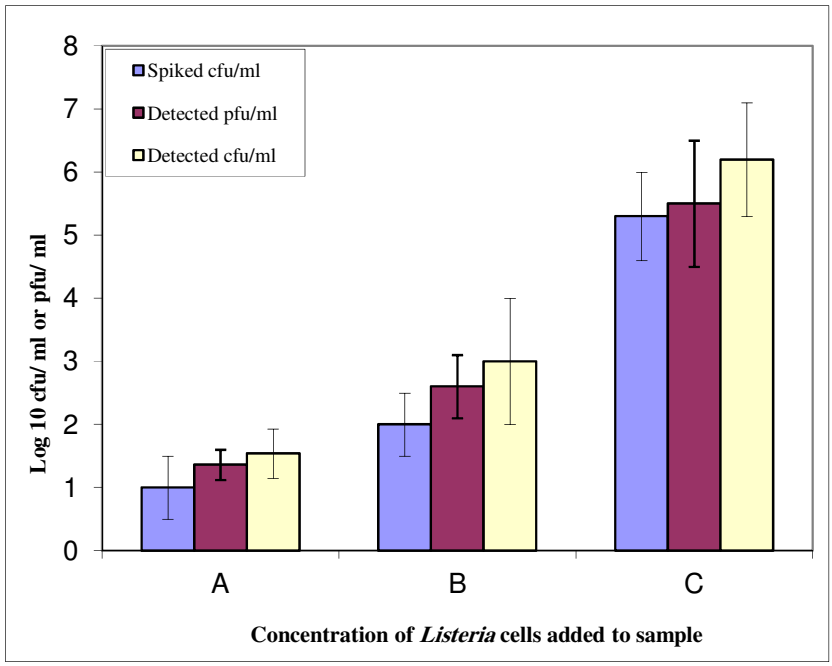
In order to determine the sensitivity and specificity of the new phage amplifcation assay in a real food system, *L. monocytogenes* WSLC 1042 was spiked into cheese to provide a contaminated sample. Slices of cheese were directly inoculated with 0.01, 0.1, 0.5 and 1.0 ml (2, 22, 135 and 2.5×10^5 CFU ml^{-1}) of *L. monocytogenes* WSLC1042 cells suspension. The phage amplification assay was conducted as described in Section 2.5 and the isolation and identification of *L. monocytogenes* after enrichment steps was performed according to standard protocols (see Fig. 6.1).

Figure 6.2 shows a comparison of the results gained using the phage amplifcation assay (pfu) when the samples were inoculated with various concentrations of cells compared to results gained when parallel samples were tested for *Listeria* by inoculation onto PALCAM and Oxford agar (cfu). The results showed that a good correlation was seen between the pfu and cfu data, but that in all cases the number of pfu detected was slightly lower than the number of cfu. Since the isolation method involves enrichment steps and dilution of samples from pre-enrichment to enrichment, it was not expected that the numbers *Listeria* cells detected would match the inoculum used. These data are provided to show that the final number of cells detected using both methods was proportional to the input inoculum size, and that there was a good agreement between the results gained using both methods.

It should be noted that the phage amplification assay result alone can also detect other *Listeria* species naturally occurring in the cheese samples and at this stage the cells giving rise to the plaques were not yet confirmed to be *L. monocytogenes*. Since the aim here was to determine whether the food matrix was compatible with the phage amplification technology, PCR confirmation of the type of cell detected was not performed.

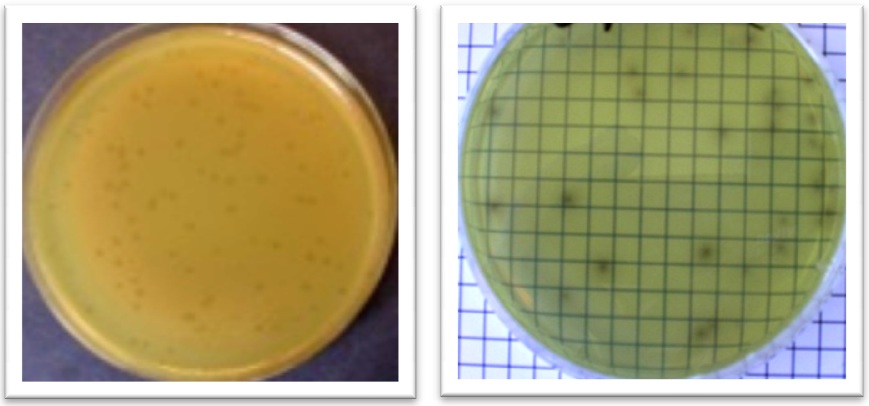
Similarly results from plating on PALCAM or *Listeria* selective agar did not facilitate the differentiation of *L. monocytogenes* from other *Listeria* species. For Oxford agar, the *Listeria* spp. colonies were black with a sunken centre and a black halo, whereas, *Listeria* spp colonies grown on PALCAM agar were grey/green in colour with a black sunken centre, and were approximately 2 mm in diameter. Colonies which were suspected to be *L. monocytogenes* from the selective plates were picked and purified by streaking them on Tryptone Soya Agar media and conformatory tests performed. Cells that were short, Gram-positive bacilli, and were haemolytic when grown on blood agar plates containing 5 % horse blood and were catalase positive were confirmed to be *L. monocytogenes* but this full identification required at least 5 days to rule out false-positive results.The confirmatory tests also revealed that some of the colonies isolated were not members of the *Listeria* genus; they were Esculine hydrolysis-positive, Gram-positive, long bacilli, but gave negative results for the PCR typing assay. These were most probably false-positive results that were able to grow on the Oxford medium.

Figure 6.2: Detection efficiency of phage amplification assay



Cheese was homogenized and samples were inoculated with different concentrations A (22 CFU ml^{-1}), B (135 CFU ml^{-1}) and C (2.5×10^5 CFU ml^{-1}) of *L. monocytogenes* WSCL 1042. The actual number of cells added was determined by viable count. Samples were then enriched in half Frazer broth and Fraser Broth was used as the secondary enrichment. Samples (0.1 ml) were taken and assayed using the phage assay (pfu). Parallel samples were tested for *Listeria* by growth on Oxford agar (cfu). Results used in this figure are the mean of three experiments ± SD.

Figure 6.3: Detection of *L. monocytogenes* using phage amplification assay and culture media



Panel A **Panel B**

Panel A; typical result of phage amplification assay (pfu data in Figure 6.2). In this assay each plaque represents the detection of one *Listeria* cell in the original sample. **Panel B**; typical result of growth of *Listeria* cells on Oxford medium (cfu data in Figure 6.2)

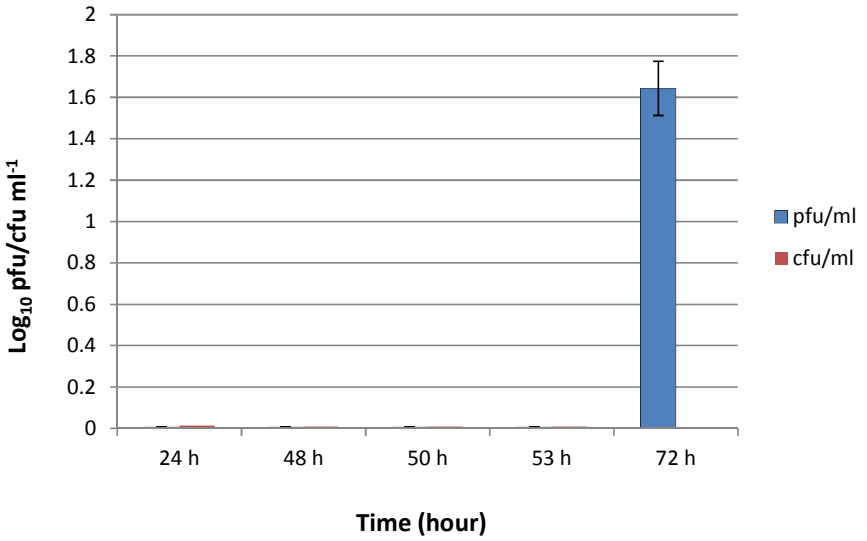
6.3.2 Earlier detection of *L. monocytogenes* In Cheese Using the Phage Amplification Assay

The aim here was to determine whether the phage amplifcaiton assay could be used to detect low levels of *L. monocytogenes* in cheese samples during the secondary enrichment stage. Stilton cheese samples were inoculated with low levels of *L. monocytogenes* (approx. 25 cells/ 25 g). Samples (0.1 ml) (phage detection assay; Fig. 6.1) were withdawn for analysis by the phage amplifcaiton assay at various incubation intervals. Parallel samples were tested for *Listeria* by growing them on Oxford agar (cfu). In this experiment, no plaques (i.e. cells) were detected for samples taken after 24, 48, 50 and 53 h from the Fraser broth enrichment samples (Fig.6.4). However plaques (40 pfu

ml^{-1}) were detected in the sample taken after 72 h. In contrast to the phage results, *Listeria* was not confirmed when samples were plated onto Oxford agar, even after 72 h. This suggested that the phage amplifcaiton assay was more sensitive than the culture method when low numbers of cells were present.

No plaques appeared in the negative control sample (no *Listeria* added to the cheese) showing that the components of the Fraser broth and cheese sample did not interfere with the virucide activity (data not shown). If interference occurs high numbers of plaques are seen in the negative control sample since the exogenous phage are not efficiently inactivated. After the selective enrichment and following dilution of the cheese enrichment, there was no contaminant seen on the phage assay plates which has been shown to be a problem when the sample being tested has a high microbial load. This results in over growth of the plates and this can obscure the plaques.

Figure 6.4: Detection of *L. monocytogenes* in Stilton cheese using phage assay



Cheese was homogenized and samples were inoculated with 25 CFU ml^{-1} of *L. monocytogenes* WSCL 1042 prepared as described in Section 2.3.2. Cells were then enriched in half Frazer broth and Fraser broth was used as secondary enrichment. 0.1 ml samples were taken from Fraser broth at various time points and assayed for the presence of *Listeria* using the phage amplification assay (pfu). Parallel samples were tested for *Listeria* by growth on Oxford agar (cfu). Results used in this Figure were the average of three experiments.

6.3.2.1 Sensitivity of Phage Assay To Detect *Listeria monocytogenes* In Cheese

Since it was clear that the phage amplification assay was able to detect *Listeria* in enrichment samples, it was investigated whether it was possible to substantially shorten the detection time required by sampling larger volumes of the enrichment culture. To do this cheese samples (25 g) were inoculated with *Listeria* (to give a final concentration of approx. 25 cells/ 25 g). Cheese

samples were homogenised in a Stomacher with 225 ml of half-Fraser broth for 2 min. Before concentrating the *L. monocytogenes* from the pre-enrichment broth, a low speed centrifugation step was applied to precipitate large food particles (80 ml samples, centrifuged at 885 ×*g* for 10 min), and then resuspended in 20 ml of Fraser broth, and then were incubated at 37 ºC continuous shaking and then to recover the bacterial cells a high speed centrifugation step was used (1 ml samples, centrifuged at 10960 ×*g* for 5 min and pellets resuspended in 0.1 ml Lambda buffer) (Section 2.6).

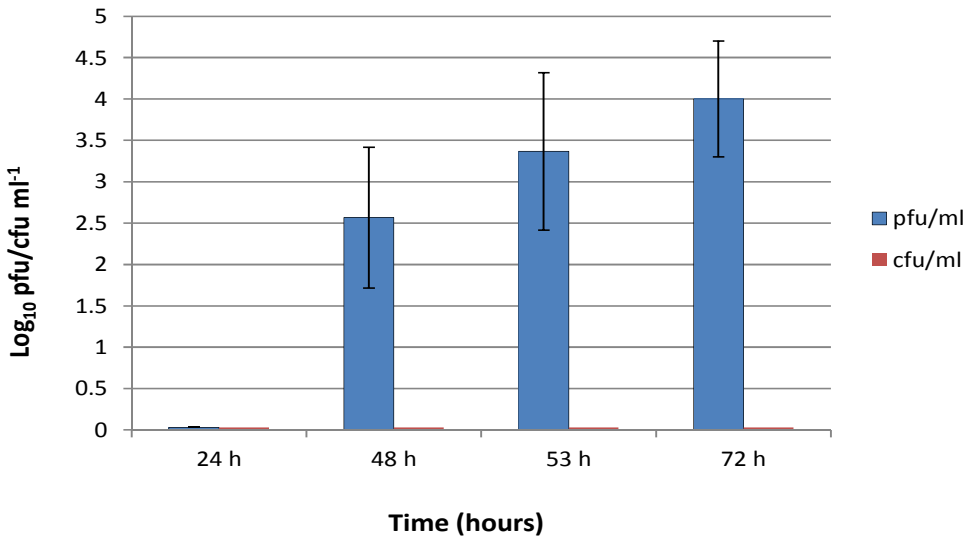
Samples were withdrawn at different time intevals for analysis using both the phage assay and by plating on Oxford selective or PALCAM media. The results are presented in Figure 6.5 and showed that *L. monocytogenes* could be detection after 48h incubation even when the cheese was contaminated with very low levels of the bacteria.

The phage amplification assay was able to detect *L. monocytogenes* in these samples after 48h, 53h and 72 h, resulting in detection of 5.0×10^2 pfuml^{-1}, 2.5×10^3 pfuml^{-1}and 2.4×10^4 pfuml^{-1}, respectively (Fig.6.5). Parallel samples were also tested for *Listeria* by growing them on Oxford agar (cfu). The obtained results showed that no colonies were detected on Oxford agar plates.

Altough these preliminary results were encouraging in terms of achieving early detection of *Listeria*, in this experiment problems were encountered with over growth of the phage amplification plates by non-*Listeria* microbes which obscured the formation of plaques (Fig. 6.6). This may have been due to the

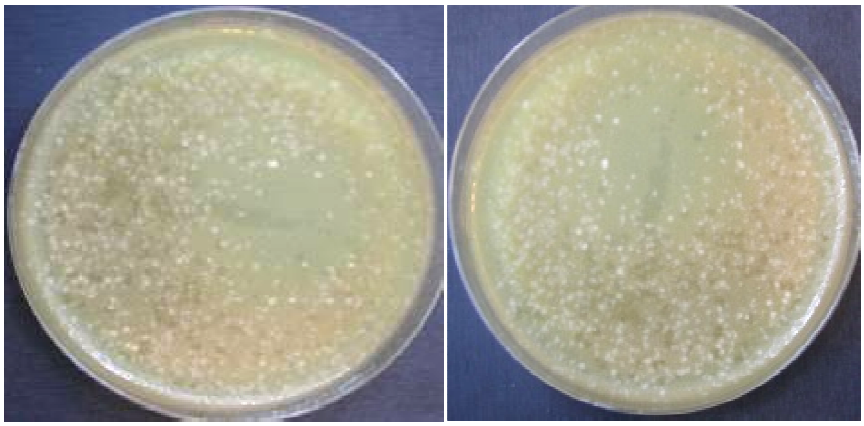
concentration of cells which form part of the natural microflora population of the original cheese during the centrifugation steps.

Figure 6.5 Detection of *L. monocytogenes* in Stilton cheese after concentration of cells



Cheese (25 g) was homogenized and samples were inoculated with *L. monocytogenes* WSCL 1042. Samples were taken and the cells were then concentrated by centrifuging. Fraser Broth was used as secondary enrichment. 0.1 ml samples were taken at the different time points and assayed using the phage assay (pfu). Parallel samples were tested for *Listeria* by growth on Oxford agar (cfu). Results used in this Figure were the average of three experiments.

Figure 6.6 Growth of competitive microbes on BHI plates used for the phage amplification assay



Examples of plates from the phage amplification assay showing growth of competitive microbes on BHI plates that obscures plaque formation when centrifugation was used to concentrate cells in the sample.

The problem of overgrowth of plaque plates by microflora present in the original sample has been described by the manufacturers of the commercial *FASTPlaque*^{TM} kit. To address this problem they provide an antibiotic supplement (NOA) that can be added to the plates to suppress this growth without affecting either the target cells or the cells that form the lawn. Hence, antimicrobial susceptibility testing was performed on *L. monocytogenes* and *L. innocua* to a range of antimicrobials to identify which ones they were resistant to. The antimicrobials tested were chosen based on the published literature concerning resistance of *Listeria* to anitbiotics and in addition the commercial NOA supplement was tested (Table 6.1). *Escherichia coli* was used as a sensitive control strain to demonstrate that the antimicrobials were effective.

While *L. innocua* was resistant to the NOA supplement, both *L. monocytogenes* and *L. innocua* were found to be resistant to both Cycloheximide (antifungal) and Fosfomycin (broad spectrum antibacterial) (Table 6.1) whereas *E. coli* was sensitive and growth was inhibited.

Table 6.1 Antimicrobial susceptibility testing of *Listeria* spp.

Organism	Antimicrobial	Effective
***L. monocytogenes* WSCL 1042**	**NAO**	Sensitive
	Fosfomycin&Cycloheximide	Resistant
***L. innocua* 11994**	**NAO**	Resistant
	Fosfomycin&Cycloheximide	Resistant
****E. coli***	**Fosfomycin&Cycloheximide**	Sensitive

NAO antimicrobial supplement (Biotec Ltd) produces a final concentration of Nystatin 50 IU ml^{-1}, Oxacillin 2 µg $ml^{-1}$and Aztreonam 30 µg ml^{-1}.NAO was prepared and used according to the manufacturer's instructions.CF antibiotic supplement (0.4 mg ml^{-1}Cycloheximide and 0.01 mg ml^{-1}Fosfomycin final concentration) was prepared as a stock solution of 40 mg ml^{-1}Cycloheximide, 1 mg ml^{-1}Fosfomycin solution in 70% ethanol. Sensitivity was determined as described in Section 2.1.17.

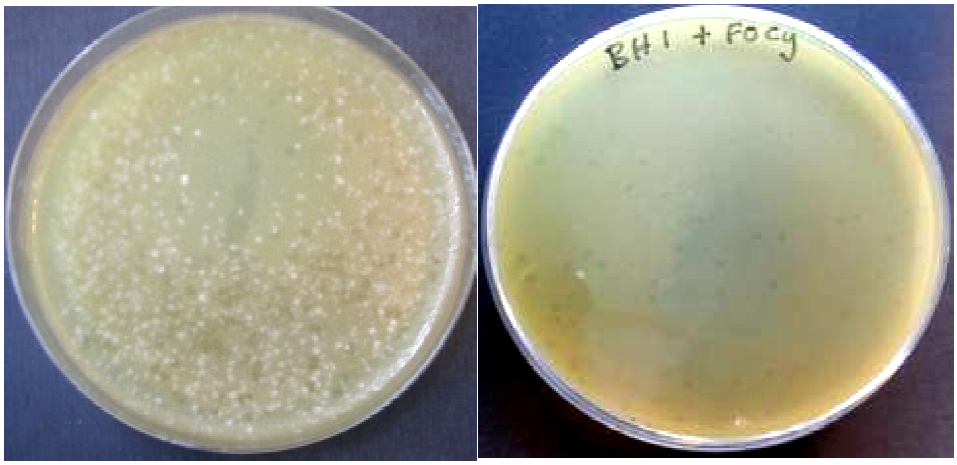
**E. coli was used as a sensitive control strain.*

Based on this result, the phage amplification assay was repeated using Protocol IV and Cheese samples inoculated with 22cfu $(25\ g)^{-1}$ *L. monocytogenes* WSCL 1042 and either Brain heart infusion agar without antibiotics or plates supplemented with Fosfomycin and Cycloheximide. The results (Fig. 6.7) showed a dramatic difference, with the overgrowth of the plates being

completely suppressed in the presence of the antibiotics and this enabled much better visualisation of the plaques.

From the results gained from the plates containing CF it was clear that *L. monocytogenes* was detected in samples taken after 48h and 53h of incubation in samples, with 4.5×10^2 pfuml^{-1} and 7.9×10^3 pfuml^{-1}, respectively, detected in these two samples (Fig. 6.8).

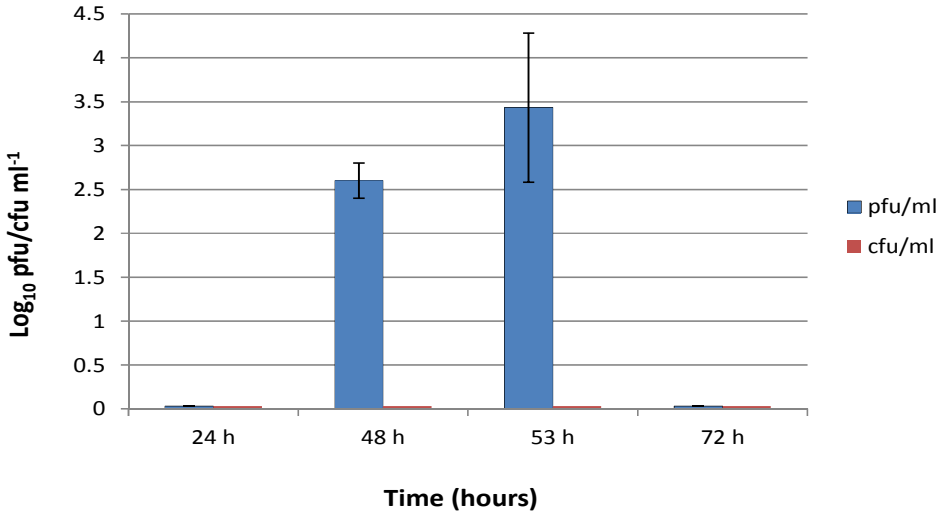
Figure 6.7 Effect of addition of antibiotics for phage detection assay



Panel A **Panel B**

Panel A; Example of plate without CF showing growth of competitive microbes on BHI plates obscures plaque formation. Panel B; Example of plate showing effect of addition of Cycloheximide and Fosfomycinwhich completely suppressed the growth of the competitive microflora.

Figure 6.8: Effect of antibiotics forphage detection assay



Cheese (25 g) was homogenized 22 cfu (approx. 1 $cfug^{-1}$) *L. monocytogenes* WSCL 1042. Cells were then concentrated by centrifuging as described above. Fraser Broth was used as secondary enrichment. Samples (0.1 ml) were taken at the different time points and assayed using the phage assay (pfu). The antibiotics (Cycloheximide and Fosfomycin) were only added to the BHI plates used at the end of the assay to plate out the samples. Parallel samples were tested for *Listeria* by growth on Oxford agar (cfu). Results used in this Figure were the average of three experiments.

6.4 Discussion

The development of a rapid and reliable test capable of detecting very low numbers of the organism in ready to eat products is of significant importance to the food industry. Detection of *L. monocytogenes* in foods is difficult as this pathogen is usually found in low numbers at the point of release when foods are being sampled. However, there is a demand for rapid methods to quantify live *L. monocytogenes* cells within a short time. The traditional culture method to detect *Listeria* in cheese products, (ISO 11290) requires enrichment and a

long incubation period. Because the phage amplification assay can detect low numbers of cells, the method developed in this study could be useful to detect low numbers of cells in cheese in shorter times than that for conventional culture methods, allowing manufactures to screen high risk products more effectively and quickly.

Soft cheese has been known as an important transmission route of human listeriosis (Makino *et al*., 2005). The ability of *L. monocytogenes* to grow at low pH environments makes it a potential hazard in milk and other dairy products (Longhi *et al*., 2003). The European Community has specified the zero tolerance directives on milk and milk for soft cheese (Longhi *et al*., 2003), accordingly, rapid and reliable test capable of detection low numbers of *L. monocytogenes* in dairy products is urgently required. Our protocols have applied the new phage amplification assay to demonstrate it is possible to detect *Listeria* in the enrichment broths when sampling a soft cheese (Stilton).

Phage A511 has been evaluated in the past as a *Listeria* biocontrol agent for control of this organism on the surface of soft cheeses during ripening (Guenther *et al*., 2009). Therefore the fact that this phage is able to infect *Listeria* cells without being inhibited by components of the cheese sample is to be expected. The technique was easy to achieve and the results were available within 48 hours and furthermore, this technique could detect higher levels of *Listeria* within 24 h. In addition, this technique is also easy to perform, uses only standard microbiological methods and does not require any expensive instrumentation therefore it is compatible with routine testing of food samples.

The microflora of cheese is very complex, and inoculation of the surface with moulds during ripening is common. However, when testing cheese samples, problems were encountered with overgrowth by non-*Listeria* microbes which obscured the formation of plaques. To overcome this, a range of antibiotics were tested, and in addition to a broad spectrum antibacterial that was reported not to affect the *Listeria* genus (Fosfomycin), an antifungal (Cyclohexamide) was used to help suppress growth of any fungi present in the sample. This combination of antibiotics completely suppressed the overgrowth of the plates.

Some colonies picked up from Oxford medium,were non-*Listeria* microbes; although they were Esculine-positive, Gram-positive they were long bacilli rather than the short rods characteristic of *Listeria*, and gave negative results using the PCR identification assay described in Chapter 5 and therefore these were considered to be false-positive results. Capita *et al*. (2001) also reported that this medium can support the growth of other organisms such as *Enterococcus* species and *Bacillus* spp.which utilise Esculin. In this case the cell morphology suggests that they were more likely to belong to the *Bacillus* spp. However this finding does mean that in this experiment, the exact counts of the number of bacteria (cfu) cannot be confirmed as the plate counts of colonies with the appropriate colony morphology might lead to an over estimation of the number of *L. monocytogenes* cells detected.

The results in this Chapter demonstrated that the phage amplification assay is a rapid, sensitive, cost-effective method to reduce the detection time of *Listeria* during enrichment procedures, that the assay is compatible with the selective broths used for *Listeria* isolations, and that by using antimicrobials to suppress

overgrowth of samples an increase in sensitivity and shortening of the time to detection could be achieved. In contrast this approach would only be more likely to increase the number of false-positive results gained using culture based methods as the number of non-*Listeria* microbes that grew on the selective media would also be concentrated in the sample.

CHAPTER 7

ISOLATION AND IDENTIFICATION OF *LISTERIA MONOCYTOGENES* IN FOODS USING THE COMBINED PHAGE-PCR ASSAY

7.1 Introduction

L. monocytogenes is ubiquitous in the environment, and its high tolerance to refrigeration at very low temperatures has been of paramount importance for its hazardous status in minimally processed foods (Norton *et al*., 2001). In many of the well-publicised outbreaks, the specific contaminated food product was identified based on both epidemiologic analysis and bacteriological confirmation of the presence of the organism in food samples (Farber and Peterkin, 1991). From this, the presence of this particular pathogen in many ready-to-eat (RTE) foods, cured meats, seafood and unpasteurised dairy foods such as cheese and milk has been found to be very problematic (Buchanan and Roland, 2000; Hwang *et al*., 2007; Lianou and Sofos, 2007; Little *et al.*, 2009; Poltronieri *et al*., 2009; Rebagliati *et al.,* 2009). Conventional methods for the detection of *Listeria* spp., including *L. monocytogenes*, have been well developed and the screening of large numbers of samples is possible, however, these methods are, nevertheless, laborious and time consuming (Duffy *et al*., 2001). Since they require a long time to produce a definitive result, results are often only gained after release of the food product (Poltronieri *et al.,* 2009).

The conventional methods for detection of *Listeria* spp involve selective enrichment and subsequent culturing on selective media and they are considered to be the gold standard test for the detection of *L. monocytogenes* (Bubert *et al.*, 1997; O'Grady *et al.*, 2009). One issue associated with detecting *Listeria* present in food samples is that many different activities are applied during food processing, including freezing, drying and heating, and this may injure the *L. monocytogenes* cells. Hence, injured cells may be undetectable when using conventional culture-based methods as the selective agents can suppress their growth (Knabel, 2002).

To overcome these limitations, various methods involving DNA probes and polymerase chain reaction (PCR), immunological techniques, molecular and phage amplification assay have been suggested (Jeršek *et al*, 2005; Stanley *et al.,* 2007). Therefore, faster alternative methods such as PCR assays have been developed to enhance or even to use instead of traditional techniques (Deneer and Boychuk 1991; Rossen *et al*., 1991; Niederhauser *et al*., 1992; Janzten *et al.,* 2006). The reliability of PCR detection methods principally depends on the presence of sufficient numbers of target molecules and also the purity of the target template (Urbanova *et al*., 2002). Although these techniques have been somewhat successful at achieving quicker detection, there have been many problems associated with direct detection of *L. monocytogenes* in food due to the presence of PCR inhibitors, the fact that they are unable to unequivocally demonstrate whether the detected cells are alive or dead (Flekna *et al.*, 2007) and because of this either false-positive or false-negative results may occur (Rip and Gouws, 2009).

In the work presented in this thesis, the phage amplification assay has been developed as a rapid method for the detection of *L. monocytogenes*. So far the assay has been shown to be compatible with the detection of *L. monocytogenes* in soft cheese, traditionally a very challenging matrix for PCR detection methods due to the high levels of PCR inhibitors. In order to determine whether the assay can be applied to a wider range of foods, and whether the presence of components of the sample matrix affected the plaque PCR identification method, experiments were designed to detect and identify *L. monocytogenes* from various kinds of foods using the combined phage-PCR assay. The foods chosen for this study were Camembert soft cheese, pasteurised milk, minced meat, turkey breast and smoked salmon, all of which have been identified as at risk foods for the presence of *Listeria*.

7.2 Isolation of *Listeria monocytogenes* from Food Samples

To detect *Listeria* in products, the standard method (ISO 11290) (Procedure IV) which requires enrichment and incubation for at least 5 days in order to achive confirmed results was used. 25 g or ml (minced meat or pasteurised milk or turkey meat or smoked salmon or Camembert cheese) were packed and stored as described earlier (Section 6.2.1). Samples were intentionally contaminated with suspensions of *L. monocytogenes* WSCL 1042 cells, and then homogenised in a laboratory homogeniser (Stomacher 400, Seward) with 225 ml of half-Fraser broth medium. Portions of 80 ml of samples were then concentrated as described in Chapter 6 (Section 6.2.1, Procedure III). Aliquots of the samples were then withdrawn for analysis with the phage assay, and

finally the results gained were compared with the results obtained using conventional bacteriological methods.

7.3 Confirmation of Cell Identity

After culture growth, samples were also streaked on Oxford agar plates and five suspected *Listeria* colonies were aseptically picked up from Oxford agar plates for further identification and confirmation (Section 6.2.1).

After the phage amplification assay the identity of the cell detected by the phage amplification assay was confirmed by using the plaque PCR method. To do this a GenElute^{TM} Bacterial Genomic DNA Kit (Sigma) was used to isolate DNA from plaques (Section 2.10.3) and 10 µl samples of the purified DNA was used in the multiplex PCR performed in 25 µl reaction mixtures. PCR products were visualized on an agarose gel (Sections 2.10.4 and 2.10.5).

7.4 Results

7.4.1 Application of the Phage-PCR Assay For the Detection of Viable *L. monocytogenes* in Food Samples

7.4.1.1 Detection of *L. monocytogenes* In Artificially Contaminated Camembert Cheese

The ability of the assay to detect *L. monocytogenes* in camembert cheese was evaluated by inoculating 25 g samples with low levels of *L. monocytogenes* WSCL 1042 to give approximately 1 cfu g^{-1}, and then the *Listeria* detection procedure was performed using the phage amplification assay. To achieve the

same early detection of the bacteria seen in the previous Chapter after 44 h of incubation the two stage centrifugation procedure to remove food particles and then concentrate the bacteria transferred into the enrichment broth described in Section 6.2.1 was used. Once the cells had been transferred to the enrichment broth samples were taken after 24, 44 and 48 h of incubation and analysed both by the phage amplification assay and by viable count on Oxford agar. Another set of samples was tested for the presence of *Listeria* in parallel using the conventional method (ISO 11290-1, 1996 method). After the enrichment steps samples of the cultures were streaked onto *Listeria* selective agar (Oxford) and further tests performed on any colonies to confirm that *L. monocytogenes* had been isolated.Tests included motility, catalase and oxidase tests and hemolytic activity on blood agar plates (see Sections 2.9).

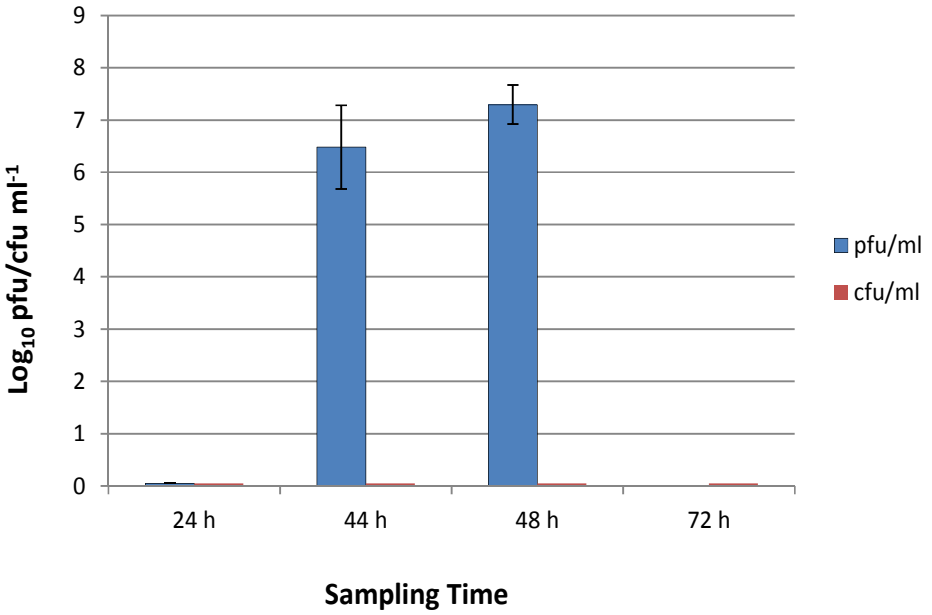
The results of the *Listeria* detetion assays are presented in Figure 7.1A and show that no cells were detectable either by the phage amplification assay or by plating the same samples on Oxford agar after 24 h of incubation. After 44 h incubation the phage amplifcation assay was able to detect *Listeria* cells represented by 7.4×10^6 pfu ml^{-1}, and this number increased to 5.9×10^7 $pfuml^{-1}$ at the 48 h sampling point.

In contrast when samples were plated on Oxford media, no growth of colonies was seen at all at these two time points and none were detected even when the sampling point was extended to 72 h. Since the same volumes were being tested in each case, this means that the phage amplification assay was better able to detect the *Listeria* cells in the sample. This could be due to sub-lethal injury of the cells which meant that they were unable to form colonies on the

selective agar plates. However at this stage from the phage assay results it could not be confirmed that the cells detected were *L. monocytogenes* rather than any other member of the *Listeria* genus.

The result for the same sample processed using the standard ISO methods showed that the level of *L. monocytogenes* used to inoculate the sample could be detected and these were confirmed to be *L. monocytogenes* after 5 days when further tests were completed.

Figure 7.1A Detection of *L. monocytogenes* in Camembert cheese using the phage amplification assay

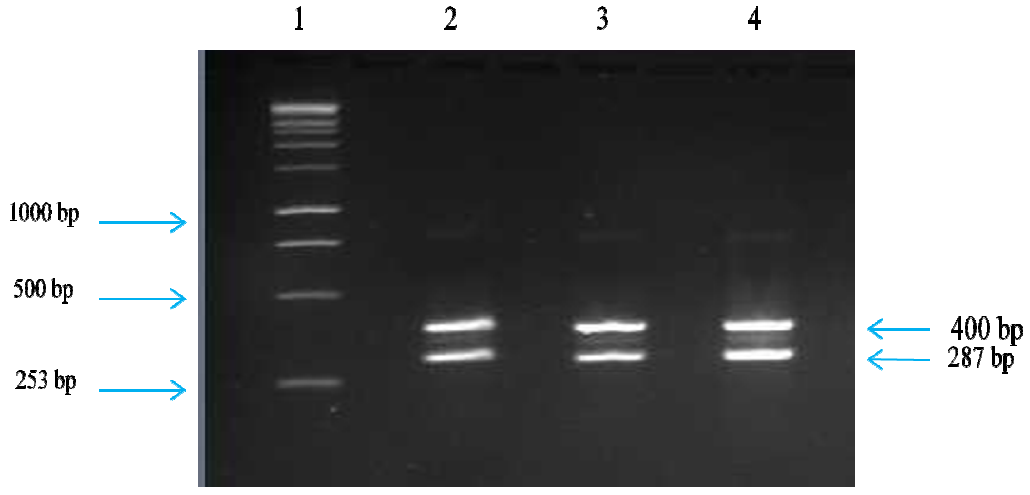


Camembert cheese (25 g) was homogenized and samples were inoculated with *L. monocytogenes* WSCL 1042 (approx.1 cfu ml^{-1}; see section 2.3.2.) Cells were then concentrated by centrifuging and transferred into Fraser broth as secondary enrichment (Section 6.2.1). Samples (0.1 ml) were taken at the different time points of secondary enrichment and assayed using the phage assay (pfu). No samples were taken for testing with

the phage after 48 h. Parallel samples (0.1 ml) were tested for *Listeria* by growth on Oxford agar (cfu). Results used in this figure were the average of three experiments.

To confirm that *L. monocytogenes* had been identified by the phage assay plaque DNA was extracted from single plaque for each experiment using the method described in section 2.10.3 and the GenElute^{TM} Bacterial Genomic DNA Kit. The PCR was performed using 10 µl of this DNA as template and the amplification conditions were as indicated in Section 2.10.4. As shown in Figure 7.1B the PCR assay amplified both the 400 bp and 287 bp bands which confirmed that it was *L. monocytogenes* that had been detected by the phage assay. The fact that the PCR assay worked for all samples tested indicated that the components of the food present in the sample tested did not interfere with the PCR assay.

Figure 7.1B Results of multiplex plaque PCR assay to identify *L. monocytogenes*.



Results of representative *Listeria* identification PCR assays using plaque DNA extracted from the Camembert cheese samples. Lane 1; molecular weight marker (Promega 1 kbp ladder).

Lanes 2-4: PCR amplification using 10 µl using plaque DNA. Samples were analysed on a 2% TAE agarose gel separated for 1.5 h at 80 V (Sections 2.10.4 and 2.10.5). Results used in this figure were three independent experiments.

For the other food samples tested in this Chapter, the experimental approach used was essentially the same. Aliquots of 0.1 ml of *L. monocytogenes* WSCL 1042 were spiked into 25g of Camembert cheese, and were added to 225 ml of half Fraser broth, homogenised by a laboratory homogeniser (Stomacher 400, Seward) at 230 rpm for 2 min. The samples were incubated at 30 °C for 22 h. 80 ml samples of the pre-enrichment samples were then centrifuged at low *g* force to remove food debris, and then at high *g* force to collect the bacterial cells. The pellet was resuspended in 20 ml Fraser broth and incubated at 37 °C. Samples (0.1 ml) were then removed from this for analysis by either the phage amplification method or by colony count on Oxford agar. In addition a parallel sample was analysed by the standard ISO method.

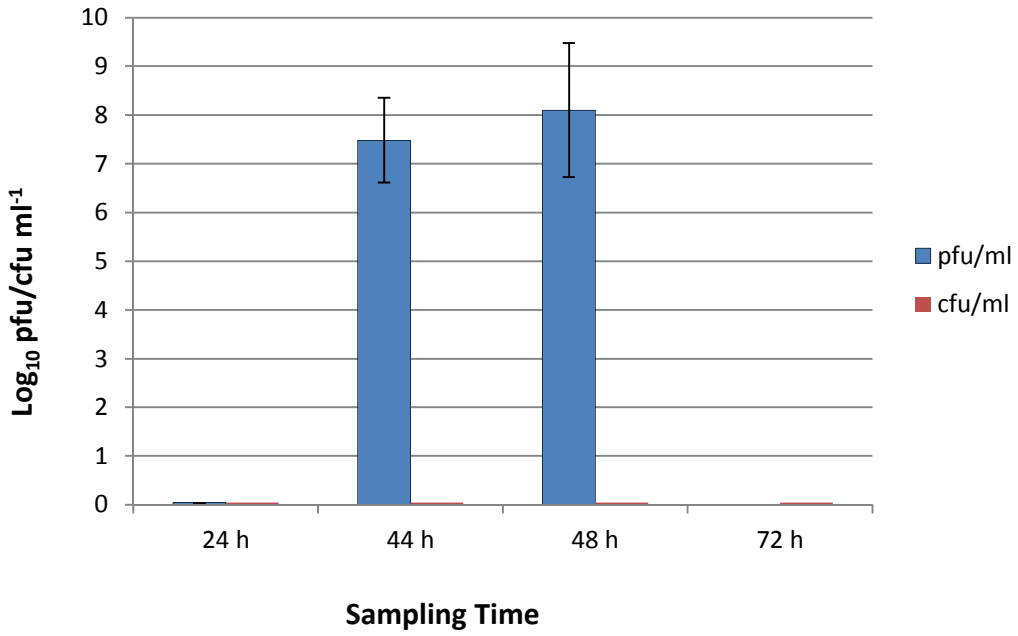
7.4.1.2 Detection of *L. monocytogenes* In Artificially Contaminated Pasteurised Milk

Pasteurised milk (25 ml) was the next real food tested. In this experiment pasteurised milk samples were artificially inoculated with 20 cfu ± 5 cells (Liquid culture of *L. monocytogenes* WSCL 1042 was prepared by inoculating a single colony into 20 ml BHI in a sterilised Pyrex conical flask and then incubating at 37 ºC with continuous shaking (200 rpm) overnight. Serial dilutions were done and 0.1 ml of dilution 10^{-7} was plated onto 5 replicates BHI agar and incubated at 37 ºC for 18 – 24 h. The number of cells was

counted as 20 cfu (± 5 cells). For comparison purposes an identitical set of pasteurised milk samples were also artificially inoculated with the same amount of *L. monocytogenes* and tested using the ISO 11290-1, 1996 method). As can be seen in Figure 7.2A, the results gained were very similar to those seen using the two cheese samples. Positive phage amplification results were gained at the 44 h, and 48 h sampling points, with the number of plaques recorded being 7.5×10^7 pfu ml^{-1} and 1.3×10^8 pfu ml^{-1}, respectively. These results suggest that detection of low concentrations of *L. monocytogenes* by this assay is reliable and feasible and is also compatible with milk samples. Simultaneously, the other set of samples were subjected to *Listeria* cells by plating the samples on Oxford agar and again no cells were detected even at the 72 h sampling point. However the presence of *L. monocytogenes* cells was confirmed after 5 days of incubation using the for ISO methods.

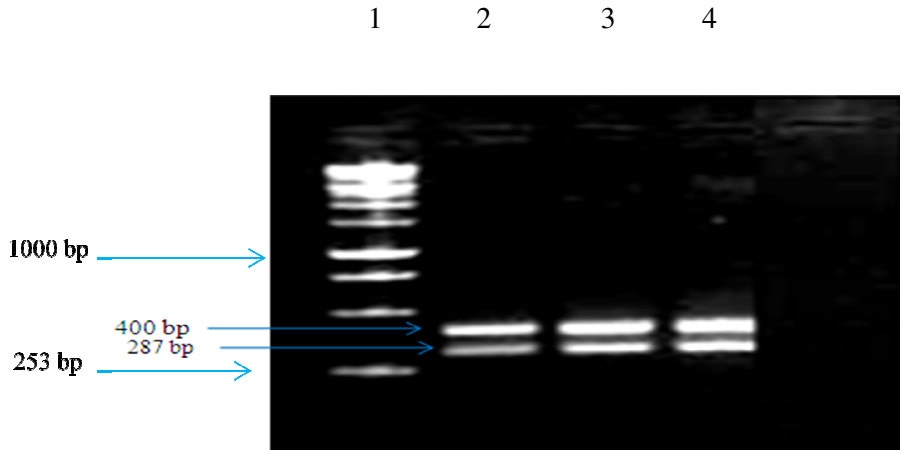
After *L. monocytogenes* cells had been processed through the phage detection assay, plaque DNA was extracted was extracted from 3 plaques using the method described in Section 2.10.2 and the GenElute™ Bacterial Genomic DNA Kit. The PCR was performed using 10 µl of this DNA as template and the amplification conditions were as indicated in Section 2.10.4. In this experiment, both 400-bp and 287bp bands, which confirm the identification of *L. monocytogenes* were obtained (Figure 7.2B).

Figure 7.2A Detection of *L. monocytogenes* in pasteurised milk using the phage amplification assay



Pasteurised milk (25 ml) was homogenized with 20 cfu (± 5 cells) *L. monocytogenes* WSCL 1042. After 22 h incubation cells were then concentrated 4-fold by centrifuging into Fraser Broth as secondary enrichment (Section 6.2.1). Samples (0.1 ml) were taken at the different time points and assayed using the phage assay (pfu). No samples were taken for testing with the phage after 48 h. Parallel samples (0.1 ml) were tested for *Listeria* by growth on Oxford agar (cfu). Results used in this figure were the average of three experiments.

Figure 7.2B Results of multiplex plaque PCR assay to identify *L. monocytogenes*



Results of representative *Listeria* identification PCR assays using plaque DNA extracted from the pasteurised milk samples. Lane 1; molecular weight marker (Promega 1 kbp ladder). Lanes 2-4: PCR amplification using 10 µl using plaque DNA. Samples were analysed on a 2 % TAE agarose gel separated for 1.5 h at 80 V (Sections 2.10.4 and 2.10.5). Results used in this figure were three independent experiments; single plaque DNA was used per experiment.

7.4.1.3 Detection of *L. monocytogenes* In Artificially Contaminated Minced Beef

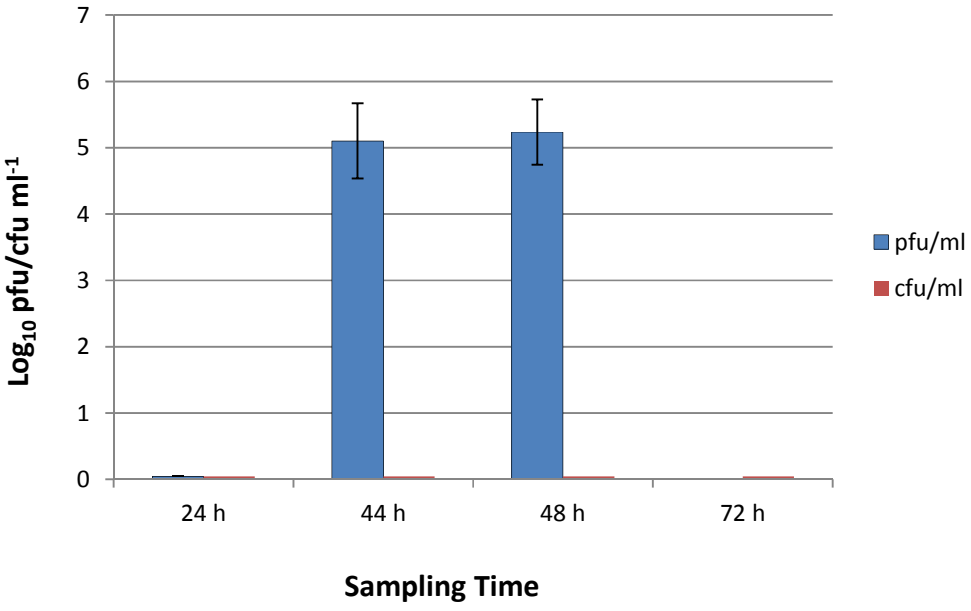
The next food tested was minced beef. As in previous sections in this Chapter the procedures applied for the detection of *L. monocytogenes* were conducted according to phage amplification procedure III (Section 6.2.1) and the standard cultural method (e.g. ISO 11290-1, 1996). In addition the multiplex plaque PCR-based method was used for the identification of *L. monocytogenes* detected by the plaque assay.

The minced beef was artificially contaminated at 20 (± 5) cfu per 25 g with *L. monocytogenes* WSCL 1042 and samples taken for detection of *L. monocytogenes* during the enrichment incubation using Fraser broth. A summary of the results obtained is presented in Figure 7.3A.

Again the concentration method allows detection of the low levels of *Listeria* added to the sample using the phage amplification assay within a total assay time of 44 hours (1.2×10^5 pfu ml^{-1} detected) and again this numner increased slightly over the next 4 hours to 2.6×10^5 pfu ml^{-1} at 48 hours.

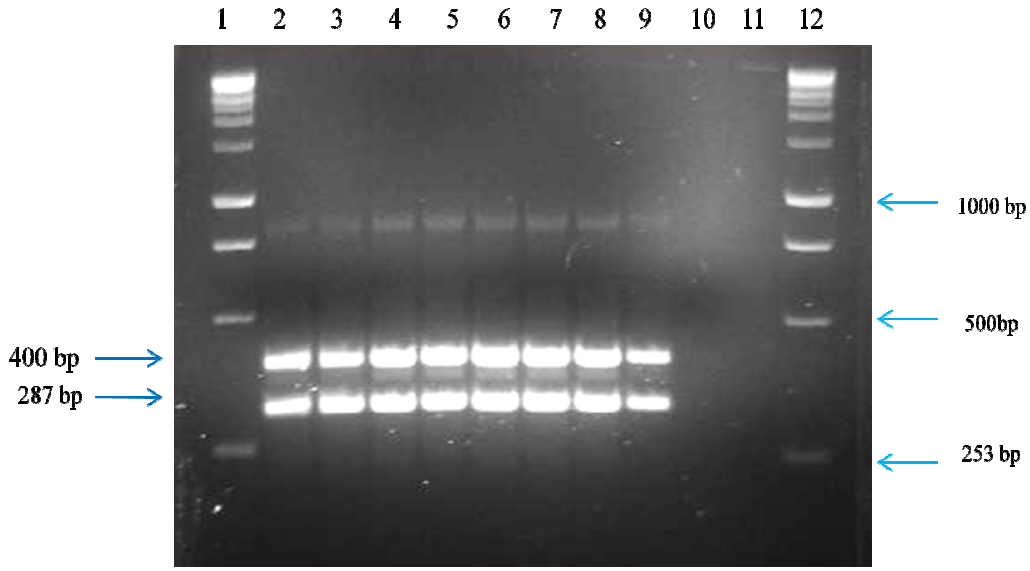
Similar to the situation with milk and Cambembert cheese, no overgrowth by contaminants was observed on the phage assay plates in this experiment despite the fact that the CF supplement was not added to the plates. Presumably the selective agents in the Fraser broth were sufficient to suppress the growth of the natural microflora present on the meat. However, this procedure still produced negative results at the 24 hour sampling point showing that growth of the *Listeria* cells during the incubation is still required for them to reach detectable levels but the presence of meat extracts in the enrichment sample does not interfere with the phage amplification detection method.

Figure 7.3A Detection of *L. monocytogenes* in minced meat using the phage amplification assay



Minced meat (25g) was homogenized with 20 cfu (± 5 cells) *L. monocytogenes* WSCL 1042. After 22 h incubation cells were then concentrated 4-fold by centrifuging into Fraser Broth as secondary enrichment (Section 6.2.1). Samples (0.1 ml) were taken at the different time points and assayed using the phage amplification assay (pfu). Parallel samples (0.1 ml) were tested for *Listeria* by growth on Oxford agar (cfu). Results used in this figure were the average of three experiments.

Figure 7.3B Results of multiplex plaque PCR assay to identify *L. monocytogenes.*



Results of representative *Listeria* identification PCR assays using plaque DNA extracted from the minced meat samples. Lanes 1 and 12; molecular weight marker (Promega 1 kbp ladder). Lanes 2-9: PCR amplification using 10 µl plaque DNA. Lane10; no DNA negative control and lane 11: *Salmonella* spp. genomic DNA (negative control). Samples were analysed on a 2% TAE agarose gel separated for 1.5 h at 80 V (Sections 2.10.4 and 2.10.5). Results used in this figure were three independent experiments.

Again the parallel samples that were tested for *Listeria* by culturing them on Oxford agar (cfu) produced no colonies, again indicating that the phage-based method is able to detect cells that are not culturable. Cells were present in the

sample that could be recovered since *L. monocytogenes* isolates were recovered on Oxford plates and PALCAM from the standard ISO isolation method and were confirmed to be *L. monocytogenes* after 5 days.

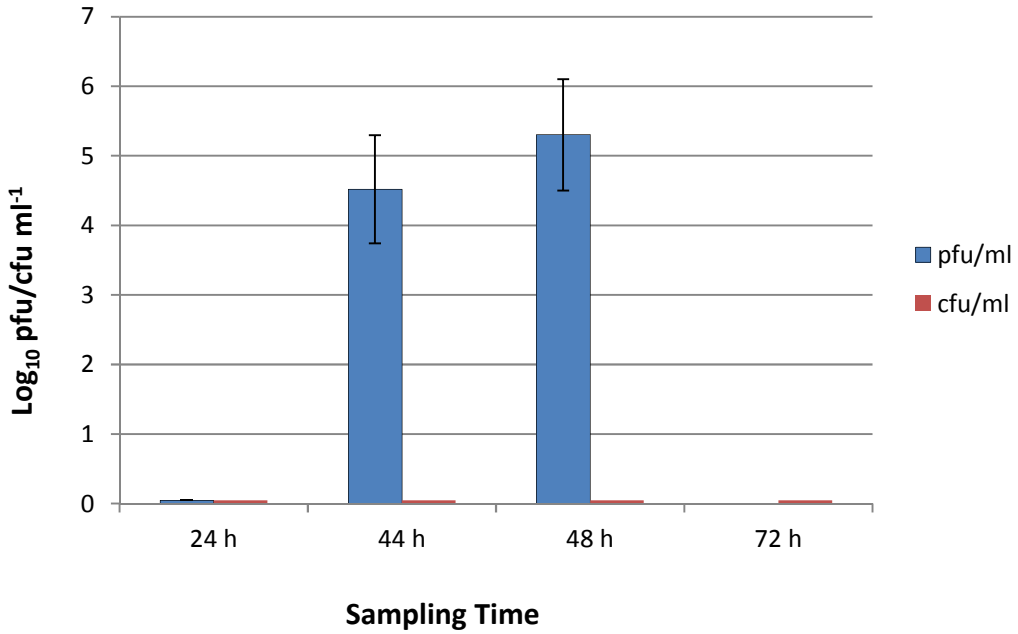
To confirm that the phage amplification assay had detected *L. monocytogenes* cells, plaque DNA was extracted using the method described in section 2.10.2. and the GenElute^{TM} Bacterial Genomic DNA Kit. The PCR was performed using 10 µl of this DNA as template and the amplification conditions were as indicated in Section 2.10.4. It can be seen from the results preented in Figure 7.3B that for the plaque samples both the 400bp and 287bp bands were obtained confirming that *L. monocytogenes* cells had been detected. However there was some evidence of non-specific amplification of a larger band of ~900 bp in this experiment suggesting that some components from the food matrix were affecting the stringency of the PCR reaction. Despite this the amplification of the target bands was not affected, with good amounts of both PCR products being generated. To show that these contaminants did not affect the overall specificity of the PCR reaction, a negative control was performed using purified genomic DNA extracted from *Salmonella* spp. as template DNA but in this case no amplification of PCR products occurred showing that the specificity was not compromised.

7.4.1.4 Detection of *L. monocytogenes* In Artificially Contaminated Turkey Breast

Next samples of raw turkey breast were used as an example of a white meat to contrast with the results from minced beef (red meat). The experimental design was the same as described in Section 7.4.1.3 except that in this case 25 g of turkey breast was inoculated with at 25 cfu per 25 g with *L. monocytogenes* WSCL 1042. The results for this experiment are shown in Figure 7.4A.

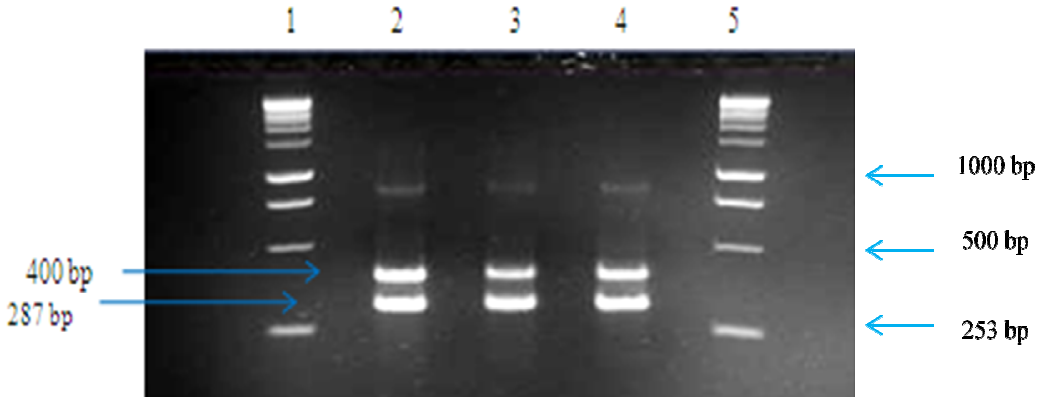
The results were remarkably in that no plaques were detected in the sample taken after 24 h but after a period of 44 h, it was observed that the phage amplification gave positive results for the detection of *Listera*. Likewise, the samples taken after further 4 hours incubation (48 h sample point) showed an increase in the number of plaques detected (4.7×10^5 pfu ml^{-1}compared to 7.5×10^4 pfu ml^{-1} at 44 h incubation). Similar to the previous experiment using minced beef, no contaminating overgrowth was observed on the phage assay plates in this experiment. Therefore the phage detection assay was able to detect *L. monocytogenes* in artificially contaminated turkey breast. The failure of these cells that were detected by the phage amplification assay to be detected when samples were plated on Oxford agar (cfu, Fig. 7.4A) was consistent with other experiments performed using real food samples. Again *L. monocytogenes* colonies were detected after 5 days on Oxford plates using the ISO method.

Figure 7.4A Detection of *L. monocytogenes* in Turkey breast using the phage amplification assay



Turkey breast (25g) was homogenized with 25 cfu and samples were inoculated with *L.monocytogenes* WSCL 1042. After 22 h incubation cells were concentrated 4-fold by centrifuging and incoulated intoFraser Broth as secondary enrichment (Section 6.2.1). Samples (0.1 ml) were taken at the different time points and assayed using the phage assay (pfu). Parallel samples (0.1 ml) were tested for *Listeria* by growth on Oxford agar (cfu). Results used in this figure were the average of three experiments.

Figure 7.4B Results of multiplex plaque PCR assay to identify *L. monocytogenes*.



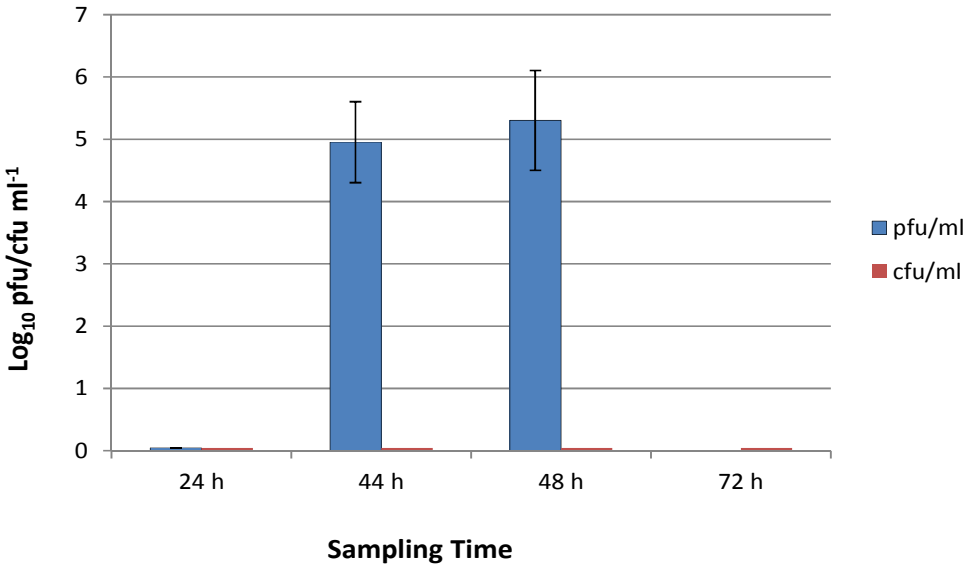
Results of representative *Listeria* identification PCR assays using plaque DNA extracted from the Turkey breast samples. Lanes 1 and 5; molecular weight marker (Promega 1 kbp ladder). Lanes 2-4: PCR amplification using 10 µl plaque DNA. Samples were analysed on a 2% TAE agarose gel separated for 1.5 h at 80 V (Sections 2.10.4 and 2.10.5). Results used in this figure were three independent experiments; single plaque DNA was used per experiment.

As before DNA was extracted using the method described in Section 2.10.2 and the GenElute^{TM} Bacterial Genomic DNA Kit. The PCR was performed using 10 µl of this DNA as template and the amplification conditions were as indicated in Section 2.10.4. Interestingly, the non-specific PCR product seen when the minced beef samples tested was again amplified when the food sample contained meat extracts, but this did not seem to compromise the abilty of the reaction components to amplify the *Listeria* spp. band of 400 bp or the *L. monocytogenes*-specific band of 287bp (Fig. 7.4B).

7.4.1.5 Detection of *L. monocytogenes* In Artificially Contaminated Smoked Salmon

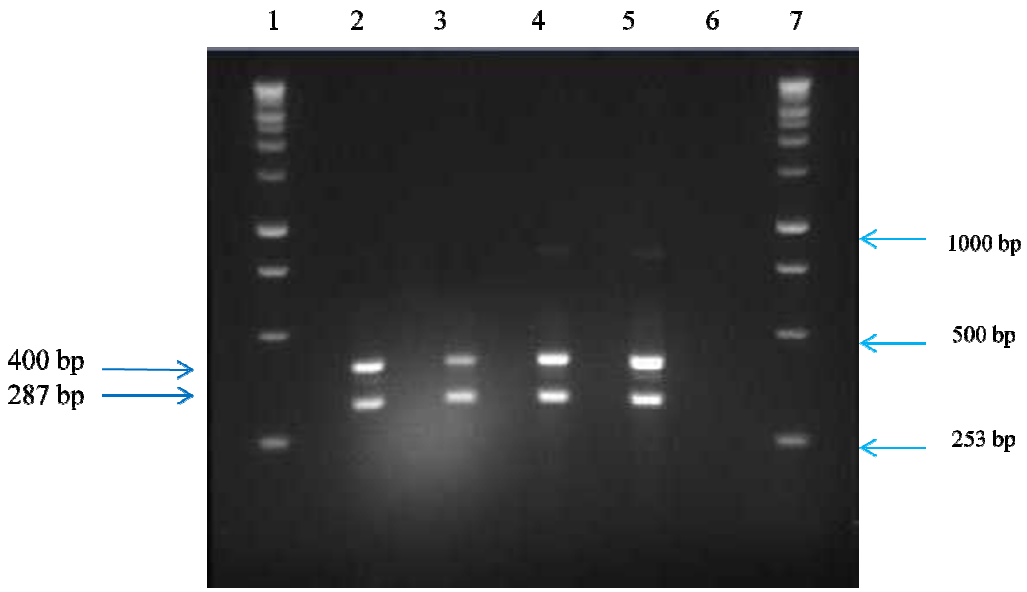
The last food sample to be tested was smoked salmon, which is known to have a particular problem with *Listeria* contamination. In this experiment 25 g of smoked salmon was inoculated with 22 cfu of *L. monocytogenes* WSCL 1042 and was homogenised with 225 ml of half-Fraser broth. After this the experimental design was as described in the previous sections in this Chapter. Figure 7.5A summarises the results obtained after phage detection assay and the results were consistent with other experiments. *Listeria* were detected after 44 h using the phage amplification assay (1.6×10^5 pfuml^{-1}) and the number of plaque detrected increased slightly at the 48 h sampling point (4.7×10^5 pfuml^{-1}). No contaminanting overgrowth of the the phage assay plates was observed and no colonies were detected when parallel samples were plated on Oxford agar, but *L. monocytogenes* was present in the samples and was detected after 5 days using the ISO method.

Figure 7.5A Detection of *L. monocytogenes* in smoked salmon using the phage amplification assay



Smoked salmon (25g) was homogenized with (22 cfu) of *L.monocytogenes*WSCL 1042. After 22 h incubation cells were concentrated 4-fold by centrifuging inoculated into Fraser Broth as secondary enrichment. Samples (0.1 ml) were taken at the different time points and assayed using the phage assay (pfu). Parallel samples were tested for *Listeria* by growth on Oxford agar (cfu). Results used in this figure were the average of three experiments.

Figure 7.5B Results of multiplex plaque PCR assay to identify *L. monocytogenes.*



Results of representative *Listeria* identification PCR assays using plaque DNA extracted from the smoked salmon samples. Lanes 1 and 7 molecular weight marker (Promega 1 kbp ladder). Lanes 2-5: PCR amplification using 10 µl plaque DNA. Lane 6; *Salmonella* spp. genomic DNA (negative control).Samples were analysed on a 2% TAE agarose gel separated for 1.5 h at 80 V (Sections 2.10.4 and 2.10.5). Results used in this figure were three independent experiments.

When plaque DNA was extracted following the detection of the *Listeria* cells using the phage amplification assay, the multiplex *Listeria* identification assay was able to produce the expected 400bp and 287bp bands as shown in Figure

7.5B and in this case the non-specific extra band at ~900 bp was only present in some of the samples tested.

7.5 Discussion

Food-borne *L. monocytogenes* is a serious threat to human health, since it is accounted for the greatest number of food product recalls and several outbreaks. It was recently commonly found in sliced deli meats, pasteurised milk, cheese and meat frankfurters (Dawson *et al.*, 2006; Mead *et al.*, 2006), pâté and soft cheese types (Health Canada, 2010). Therefore, novel quicker and more specific techniques and strategies to combat this opportunistic pathogen in various foodstuffs are urgently needed. Hence developing quicker methods for detection of *L. monocytogenes* have attracted the consideration of many researchers over the past few decades, though the methods that are capable of detecting low numbers of this pathogen are often limited.

When the ability of the phage amplification assay to detect *L. monocytogenes* was tested in a wide range of foodstuffs known to be frequently contaminated by this pathogen it was shown to be able rapidly and reproducibly to detect viable *L. monocytogenes* cells inoculated into the foods at low numbers in a relatively shorter time compared to conventional culture methods. These results were obtained by developing a centrifugation method to concentrate and separate the cells from components of the food samples prior to enrichment. The developed methods could be used in combination with the combined phage-PCR strategy described here or could be used as a complementary

technique to obtain quicker identification of *L. monocytogenes* using other detection methods.

Interestingly the problem encountered when using the concentration method when sampling Stilton cheese was not seen when other foods were tested. The selective agents in Fraser broth are lithium chloride which inhibits the growth of most enterococci, Nalidixic acid which inhibits DNA gyrase in cells and affects DNA replication in bacteria and yeast that are sensitive to this antibiotic, and Acriflavine – a DNA intercalating agent which inhibits the growth of sensitive Gram-positive bacteria and is also an antifungal agent. Clearly while these were able to suppress the growth of the competitive microflora present in most of the food samples, the Stilton cheese seemed to contain organisms that were resistant and were able to grow in the secondary enrichment broth.

It was observed that there was a difference between the number of cells detected by the phage assay (pfu) and by viable count on Oxford agar (cfu results). The cells being sampled are being grown in Fraser broth that contains selective agents and, although resistant, these may stress the *Listeria* cells as well as inhibiting the growth of competitive organisms. It has been previously reported that the selective agents present in Fraser broth reduce the sensitivity of *Listeria* detection methods due to sub-lethal injury of the cells (Lammerding and Doyle, 1989; Vaz-Vehlo *et al*., 2001; Supanivatin *et al.,* 2012) and the findings in this study support this conclusion.

The use of centrifugation was also used to improve detection. In essence this involves compacting bacteria into a pellet, causing collisions against each other, which may result in surface components being stripped off or compressed from shear force on the bacterial cell surface. This may lead to damage of the cell surface and interior structures, including DNA, and such damage caused by centrifugation has been previously reported (Peterson *et al*., 2012). This could contribute to injury of cells reducing the ability to form colonies.

Interestingly, in contrast, the sub-lethal injury did not appear to affect the phage infection process, and of course the *L. innocua* cells that form the lawn are not grown in the presence of selective agents and so are not experiencing the same stress when added to the plates. The results presented also show that the positive results were gained at the 44 hours, followed by a more gradual increase after 4 hours. The interaction of phage with *Listeria* cells occurred by specificity of adsorption, which is dependent on the structural properties of receptors on the *Listeria* cell surface. Therefore the increase in the plaque forming units may not in fact represent growth, but the fact that the cells are producing increasing amounts of the required receptor, and hence explains the increase in the plaques formed.

The results gained in this Chapter indicated that *Listeria* cells could be detected using the phage amplification assay in minced meat, turkey breast, smoked salmon, Camembert and Stilton cheeses and in pasteurised milk. When these experiments were performed a negative control sample was prepared to which no *Listeria* cells were added to show that the virucide was inactivating all the

phage that did not infect host cells. As no plaques were found in these negative control samples it was concluded that the virucide was not affected by any of the components of the food matrix that were present in the enrichment samples. The broad host range of the A511 phage means that the identity of the cells detected in the phage assay needed to be confirmed by using further molecular techniques as described in Chapter 4. The GenElute^{TM} bacterial genomic DNA kit was found to be able to purify plaque DNA reliably as a template for the multiplex PCR assay. The fact that the multiplex PCR identification assay targets the16S rRNAgenes, and there are several copies of this in each *Listeria* cell, is probably the reason why the single target cells in the plaques were so consistently detected by the PCR assay. If single copy genes had been targeted it is possible that the PCR assay would not have been so successful.

Even though the multiplex PCR reaction was successful it was clear that when the PCR assay was performed for some samples, it appeared that some food components present in the enrichment sample affected the specificity of the PCR and an additional band of ~900 bp was seen in some cases. Complex food matrices contain high amounts of protein, calcium ions, fats and may have more sources of different organisms which could interfere with DNA in the sample. However when looking at the sampling procedure, the actual amout of food material that would be carried over into the final plaque is very small (10 µl sample taken from 0.1 ml of a 4-fold concentrated 80 ml sample of enrichment broth, and this 80 ml sample represents only approx. 1/3 of the total food sample added to the Fraser broth). Hence it is probably unlikely that this is the cause of the interference seen in these PCR assays. More likely this

larger band represents a PCR product produced because of variation in the PCR conditions resulting from the use of either different PCR blocks, or even the use of different positions within one PCR block. It is possible that under certain PCR conditions the formation of this larger product is allowed by a small reduction in the stringency of the PCR conditions. Since the gene targeted by the PCR assay is part of the 16S rRNA gene sequence, and these genes are tandemly arranged on the chromosome, this larger band could represent the amplification of a product that spans two 16S rRNA genes. However, as the size of the bands produced by the assay is diagnostic for the detection of *Listeria*, and the amplification of the expected bands was not affected, the appearance of this large band did not affect the abililty of the PCR assay to identify the cell detected.

To conclude, the overall time required for final detetion and identification of the combined phage-PCR assay developed here was 48 h (e.g. 44 h until the phage assay could detect the *Listeria* cells and a further 4 h to complete the PCR assay, including DNA extraction and PCR amplification). This was significantly shorter than the conventional culture-based method (ISO 11290) and the specificity of the phage and PCR means that the problems seen using PALCAM and Oxford media when a significant number of non-*Listeria* microbes (Esculine positive) were found are avoided when using the combined phage-PCR method.

CHAPTER 8

CONCLUSIONS AND FUTURE DIRECTIONS

The incidence of *L. monocytogenes* in different food products has been a worldwide problem in the food industry as the bacterium is able to tolerate, survive and grow in diverse environmental conditions. Therefore the occurrence of this human pathogen in foods that will support growth of the organism is considered as being a significant risk for human listeriosis. However due to its widespread presence in different raw materials, the occurrence of this pathogen in low numbers in some raw and minimally processed/Ready to Eat (RTE) foods may be unavoidable (Premaratne *et al*., 1991) and therefore there is a continuing need to test high risk foods for the presence of the organisms.

Different countries specify different limits for the presence of *L. monocytogenes* in RTE foods. The USA has a 'zero tolerance policy' for *L. monocytogenes* in any RTE food and manufacturers must demonstrate an absence from 25 g of sample (< 0.04 $cfug^{-1}$). Until recently this was applied to all RTE foods, but because of the practical difficulties in ensuring that all foods met this criteria, a change in guidance was issued (Health Canada, 2011), that indicated that this stringent test would only be applied to foods that can support significant growth of *Listeria* during cold storage. Any food that allows more than an average of 0.5 log $cfug^{-1}$ increase in *L. monocytogenes* levels before the end of shelf life is considered a food in which growth can occur. However where growth was not likely to occur, such as in frozen foods, those with a pH

less than 5.0 or those with a water activity of less than 0.92, the limits (http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodProcessingHACCP/ucm073110.htm?utm_campaign=Google2&utm_source=fdaSearch&utm_medium=website&utm_term=listeria&utm_content=3) for this organism have been increased to less than 100 cfu g^{-1}. This is in line with the European Union regulations (2007), which allow up to 100 $cfug^{-1}$ at the end of shelf life for RTE foods. The exception to this is foods for infants or those for special medical purposes because infants and immuno-compromised patients have a higher risk of infection. However since foods that do support growth have a short shelf life, testing of product needs to be rapid. Product recalls are always damaging for a company, and therefore if a problem is detected the manufacturers would like to know that a product has failed the microbiological testing as soon as possible.

As described in this thesis, standard microbiological *L. monocytogenes* testing using ISO 11290-1 takes up to 5 days. To reduce this time rapid methods have been developed such as the BAX system (Oxoid). This is used to detect members of the *Listeria* genus in environmental samples but is not appropriate for food samples. This may be because it uses a PCR assay to detect *Listeria* cells after the samples have been enriched, and components of food are known to inhibit PCR detection assays. Therefore it is recommended for environmental swab and sponge samples and uses a modified single enrichment step. This reduces the time required for the assay to 28-32 h but the test only indicates the presence of *Listeria* spp. and further culture and confirmatory tests are required to determine which species is present. Since *L.*

monocytogenes is often present in mixed populations with other members of the *Listeria* genus, these species can be used as an indicator that *L. monocytogenes* could be present. While this is useful when testing environmental samples, for food samples it is important to know whether or not the organism detected in a food sample is pathogenic.

A commonly used rapid method is the VIDAS system produced by Biomerieux. This is an automated antibody-based system that has been marketed for some time and until this year was also only able to detect *Listeria* spp., but could be used for both environmental and food samples. However in 2012 a new VIDAS system was launched that detects both *Listeria* spp. and *L. monocytogenes* species specifically. The time to detection using the VIDAS LDUO is still 46-50 hours as two enrichment steps are required.

Since the detection of low levels of *L. monocytogenes* in foods presents a significant challenge to food microbiologists, therefore this work has mainly focused on optimising and evaluating a novel combined phage-PCR procedure for the identification of viable *L. monocytogenes* in various foodstuffs. Other rapid methods designed to detect *L. monocytogenes* faster have been reported and have been reviewed by Zunabovic *et al.* (2011) and Jadhave *et al*. (2012). Both reviews report that the same problems are seen with many of these published methods. These are (1) low sensitivity of the technique leading to a requirement for long enrichment period to achieve detection and (2) inhibition of PCR reactions by food components.

The features of the combined phage-PCR assay developed in this work help address these problems in that the enrichment times were shortened by using centrifugation to concentrate cells. The broad host range phage A511was used to develop the detection assay as it has previously been shown to be able to infect a wide range of *Listeria* isolates in foods (Loessner *et al*., 1997; Guenther *et al*., 2009). A wide range of compounds were screened for virucidal activity against the phage which would not cause any injury to the infected target bacteria. The results indicated that A511 was sensitive to virucide treatment using extracts from tea leaves. Tea extracts had previously been found to be a good virucide by De Siqueira *et al.* (2006). The ability to detect *Listeria* using a phage amplification assay is in agreement with earlier findings reported by Stewart *et al*. (1998) who indicated that this technology could be used to detect *Listeria.* However in this case the phage used was not broad range enough to detect all strains of *L. monocytogenes.* In accordance with Stanley *et al.* (2007), it was found that the plaque PCR method could be used in combination with bacteriophage. In this experiment, the identity of the cells detected were confirmed using PCR amplification of signature sequences from the phage plaque. Moreover, the extraction method developed here was rapid, simple and very efficient and it was shown that using a DNA extraction kit was a better method to purify DNA from plaques.

However reproducible amplification of single copy genes can be difficult as it is hard to have 100 % efficient DNA extraction. The PCR method used in this experiment was based on amplification of 16S rDNAsequences according to the method of Somer and Kashi (2003) and therefore targets multi-copy genes

which make it more likely that the target DNA will be extracted. In addition using the phage to extract the DNA from a single cell embedded in agar at the end of the assay seems to remove the inhibitory effects of the food components when a range of food types were tested. Although some effect was seen when meat or fish products were tested so that additional bands were amplified in the assay, this did not affect the ability of the primers to amplify the correct PCR products.

One of the drawbacks of using PCR assays alone for detection of pathogens is that PCR detects both viable and non-viable cells, whereas, when it is combined with the phage assay, the cells had to be viable in order them to be detected. This advantage is important pathogenic bacterial detection (e.g. when testing heat treated or pasteurised foods that may contain dead cells that are destroyed during food production). The other advantage of this integrated phage PCR assay over other PCR-based detection methods is its simplicity of sample preparation, since the phage selectively lyse the target cell and the DNA from the single host cell is held in a fixed place so it is easy to find.

Centrifugation was used as a simple and rapid way to concentrate and separate cells from sample prior to being detected with the phage. Using centrifugation to concentrate the cells did create a problem when working with Stilton cheese as the natural microbes present were also concentrated and this caused overgrowth of the phage assay plates when no selective agents were added to them. Adding antimicrobials to suppress the overgrowth of samples to the assay plates overcame this problem. The use of *Listeria* selective plates in the phage assay was considered as an alternative, but for good visualisation of

plaques the plates need to be quite clear and the selective agents in PALCAM and Oxford agars are not clear enough to allow the plaques to be seen.

The use of differential centrifugation to remove large food particles before concentrating cells from a sample has been described before (reviewed by Ruban *et al.*, 2011). This review also describes other methods that can be used to separate and concentrate bacteria present in food samples.

So although centrifugation was used in this study, future work could investigate other methods such as filtration or immunomagnetic separation using antibody coated magnetic beads. However filtration of food samples can be difficult, and immunomagnetic separation is generally more efficient in small volumes, and the centrifugation method used here did seem to be effective.

Niederhauser *et al.* (1992) described a method using centrifugation at 100 x *g* to eliminate large food particles followed by a centrifugation at 3000 x *g* to collect the bacteria from both primary and secondary enrichment cultures. Using this method they improved PCR detection limits for *L. monocytogenes* in meat samples 1000-fold to 103 cfu *L. monocytogenes* per gram of meat, although this was still too high to be useful for detection of *Listeria* in foods that allow growth.

Interestingly this group also found that some cells they could detect by PCR-based methods were not able to form colonies on selective agar. They

suggested that the cells in the food sample were injured. This is consistent with the findings in this work that cells could be detected by phage, and the DNA detected in a plaque, but they were unable to form colonies when plated on selective media.

The ability of phage to infect cells that cannot form colonies has been described before when researchers have been using reporter bacteriophage. These phage are modified and contain reporter genes that produce a signal when the phage infects a cell. Awais *et al.* (2006) used a GFP-reporter phage to detect viable but non-culturable *E. coli* cells and Birmele *et al.* (2008) showed that a reporter phage was able to infect *E. coli* cells that were sub-lethally injured. So it is possible that phage can infect and replicate in any injured cells present in the sample that are unable to form colonies when plated on selective media. This suggests that the phage-based detection method could be more sensitive than conventional culture methods if the cells present in a food sample have been injured. Moreover in processed foods *Listeria* cells can be injured by processing treatments such as freezing and heating. In conventional culture-based testing long pre- and secondary enrichments are used to amplify these low initial numbers to detectable levels. Further investigations could be carried out to prove that the difference in pfu and cfu results seen here are due to sub-lethal injury of cells during the enrichment process.

When developing the assay it was considered that the state of the cells being detected could affect the ability of the phage to infect them, especially if the cells form capsules. This is more likely for environmental samples when they are growing on surfaces with little nutrient available rather than in foods. To

improve the understanding of the interactions between the phage with its host cell, cells were grown in minimal media. It was found that the efficiency of phage infection was reduced by about 1 $\log_{10}$. This reduction was about the same as the lowest level efficiency of infection seen when the ability of phage A511 to infect other strains and species of *Listeria* was tested. These two factors could reduce the sensitivity of the phage-based assay if the phage cannot infect the target cells efficiently. However when performing the assay the number of phage added to the sample is very large compared to the number of cells and this may overcome the problem. One point that supports this is that *Listeria* phage A511 has been shown to be a useful tool for control of *Listeria* contamination of foods, in that adding high number of phage to the food was able to suppress the growth of natural *Listeria* contaminants (Klumpp *et al*., 2008), which suggests that these phage will be able to infect the *Listeria* cells present at low levels in food samples and environments. Whether the assay is able to detect all strains of *Listeria* in foods, and is able to detect *Listeria* that have naturally contaminated foods needs to be evaluated in further studies, but there was not time to complete these studies as part of this thesis.

The findings in this study have investigated the potential for the detection of *Listeria* cells using a phage amplification assay method which is specific and low cost and has the advantage of simplicity of. In order to prove the feasibility of this concept, the efficiency of the new developed assay was tested using Stilton cheese. The results obtained have shown that this method was able to detect low numbers of cells in only two days compared to at least five days when using conventional standard methods. More importantly, another finding

from this study was that the phage assay is compatible with the standard selective broths used for *Listeria* isolations and therefore it does not require any specialised equipment or materials that are not routinely used for the isolation and identification of *L. monocytogenes*.

Therefore the results obtained also showed that this new technique could be a good alternative for rapid detection of *L. monocytogenes* in various foodstuffs to provide early indication that a product is contaminated or it would allow rapid screening for food products prior to release from the factory. The limit of detection achieved was 20 (±5) cfu per 25 g and the combined phage-PCR method achieved specific identification of *L. monocytogenes* within 2 days, compared to 5 days when using conventional culture methods. Therefore, it can be concluded that the successful integrated phage detection assay with the confirmatory PCR of plaque DNA is capable of detecting viable *L. monocytogenes* extracted from infected food in just 2 day (i.e. 48 h) with highly specific and is a practical and cost-effective method.

Overall, the application of bacteriophage is definitely promising in rapid detection of pathogenic bacteria in various foodstuffs. The future work in this area will be focusing on further development of PCR primers that would be used in distinguishing the rare serotypes. Future research would also include more work to see if the method can be used to detect low levels of pathogens in real foods and in food processing environments by completing surveys comparing standard culture to the integrated phage-PCR method so that the value of this new assay can be fully evaluated.

REFERENCES

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

Abdelgadir, A., Srivastava, K., and Reddy, G. (2009) Detection of *Listeria monocytogenes* in ready-to-eat meat products. *American Journal of Animal and Veterinary Sciences* 4, 101-107.

Abrahão, W.M., Abrahão, P.R., Monteiro, L.B., and Pontarolo, R. (2008) Occurrence of *Listeria monocytogenes* in cheese and ice cream produced in the State of Paraná. *Brazilian Journal of Pharmaceutical Sciences* 44, 289-296.

Ackermann, H.W. (2003) Bacteriophage observations and evolution. *Research in Microbiology* 154, 245–251.

Ackermann, H.W. (2007) 5500 Phages examined in the electron microscope. *Archives of Virology* 152, 227-243.

Ackermann, H.W., and DuBow, M.S. (1987) Viruses of prokaryotes: Viruses of prokaryotes, Vol. I. General properties of bacteriophages. In: CRC Press, Boca Raton.

Ahn, Y.J., Kawamura, T., Kim, M., Yamamoto, T., and Mitsuoka, T. (1991) Tea polyphenols: selective growth inhibitors of *Clostridium* spp. *Agriculture and Biological Chemistry* 55, 1425-1426.

Almeida, P., and Almeida, R. (2000) A PCR protocol using *inl* gene as a target for specific detection of *Listeria monocytogenes*. *Food Control* 11, 97-102.

Anon, (1999) Centers for Disease Control and Prevention. Update: multistate outbreak of listeriosis—United States, 1998–1999. MMWR Morb Mortal Wkly Rep. 47, 1117–8.

Ardö, Y. and Petterssona, H.E. (1988) Accelerated cheese ripening with heat treated cells of *Lactobacillus helveticus* and a commercial proteolytic enzyme. *Journal of Dairy Research* 55, 239-245

Arslan, S. and Özdemir, F. (2008) Prevalence and antimicrobial resistance of *Listeria* spp. in home made white cheese. *Food Control* 19, 360-363.

Atterbury, J.A., Connerton, L.P., Dodd, E.R.C., Rees, E.D.C. and Connerton, F.I. (2003) Application of host-specific bacteriophages to the surface of chicken skin leads to a reduction in recovery of *Campylobacter jejuni*. *Applied and Environmental Microbiology* 69, 6302-6306**.**

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

Autio, T., Hielm, S., Miettinen, M., Sjöberg, A.M., Aarnisalo, K., Björkroth, J., Mattila-Sandholm, T. and Korkeala, H. (1999) Sources of *Listeria monocytogenes* contamination in a cold-smoked rainbow trout processing plant detected by pulsed-field gel electrophoresis typing. *Applied and Environmental Microbiology* 65, 150-155.

Awais, R., Fukudomi, H., Miyanaga, K., Unno, H. and Tanji**,** Y. (2006) Recombinant bacteriophage-based assay for the discriminative detection of culturable and viable but non-culturable *Escherichia coli* O157:H7. *Biotechnology Progress* 22, 853–859.

Aznar, R. and Alarcón, B. (2003) PCR detection of *Listeria monocytogenes*: a study of multiple factors affecting sensitivity. *Journal of Applied Microbiology* 95, 958-966.

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

Bansal, N.S., McDonell, F.H.Y., Smith, A., Arnold, G., and Ibrahim, G.F. (1996) Multiplex PCR assay for the routine detection of *Listeria* in food. *Internationa Journal of Food Microbiology* 33, 293-300.

Barbalho, F.T., Almeida, F.P., Almeida, C.R., and Hofer, E. (2005) Prevalence of *Listeria* spp. at a poultry processing plant in Brazil and a phage test for rapid confirmation of suspect colonies. *Food Control* 16, 211-216.

Bayles, O.D., Annous, B.A. and Wilkinson, J.B. (1996) Cold stress proteins induced in *Listeria monocytogenes* in response to temperature downshock and growth at low temperatures. *Applied and Environmental Microbiology* 62, 1116 – 1119.

Becker, B., Jordan, S. and Holzapfel, W.H. (2005) Rapid and specific detection of *Listeria monocytogenes* in smoked salmon with BAX®-PCR. *Food Control* 16, 717-721.

Becher, B., Schuler, S., Lohneis, M., Sabrowski, A., Curtis, W.D. and Holzapfel, H.W. (2006) Comparison of two chromogenic media for the detection of *Listeria monocytogenes* with the plating media recommended by EN/DIN 11290-1. *International Journal of Food Microbiology* 109, 127–131.

Berrada, H., Soriano, J.M., Pico, Y., Manes, J. (2006) Quantification of *Listeria monocytogenes* in salads by real time quantitative PCR. *International Journal of Food Microbiology* 107, 202–206.

Berrang, M., Meinersmann, R., Northcutt, J. and Smith, D.P. (2002) Molecular characterization of *Listeria monocytogenes* isolated from a poultry further processing facility and from fully cooked product. *Journal of Food Protection* 65, 1574-1579.

Bhaduri, S. and Cottrell, B. (1998) A simplified sample preparation method from various foods for PCR detection of pathogenic *Yersinia enterocolitica*: a possible model for other food pathogens. *Molecular* and *Cellular Probes* 12, 79 – 83.

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

Birmele, M., Ripp, S., Jegier, P., Roberts, M,S. and Garland, J. (2008) Characterization and validation of a bioluminescent bioreporter for the direct detection of *Escherichia coli*. *Journal of Microbiological Methods* 75, 354–356.

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

Bortolussi, R. (2008) Listeriosis: a primer. *Canadian Medical Association Journal* 179**,** 795–797.

Bosilevac, J.M., Guerini, M.N. and Koohmaraie, M. (2009) Increased detection of *Listeria* species and *Listeria monocytogenes* in raw beef using the assurance GDS molecular detection system with culture isolation. *Journal of Food Protection* 72, 674 – 679.

Botsaris, G., Slana, I., Liapi, M., Dodd, C., Economides, C., Rees, C., Pavlik, I. (2010) Rapid detection methods for viable *Mycobacterium avium* subspecies *paratuberculosis* in milk and cheese. *International Journal of Food Microbiology* 141, S87-S90.

Brett, M.S., Short, P., McLauchlin, J. (1998) A small outbreak of listeriosis associated with smoked mussels. *International Journal of Food Microbiology* 43, 223–229.

Broome, C.V., Gellin, B. and Schwartz, B. (1990) Epidemiology of listeriosis in the United States. In*: Foodborne listeriosis*. pp 61-65. (Editors: Miller, J.A., Smith, L.J. and Somkuti, A.G.). Society for Industrial Microbiology. Elsevier Science Publishing, Inc., New York.

Brüssow, H. (2001) Phages of dairy bacteria. *Annual Review of Microbiology* 55, 283-303.

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

Bubert, J.R., Schnitzler, N., Schonberg, A., Goebel, W. and Schubert, P. (1997) Isolation of catalase-negative *Listeria monocytogenes* strains from Listeriosis patients and their rapid identification by anti-p60 antibodies and/or PCR. *Journal Clinical Microbiology* 35, 179-183

Buchanan, R. and Roland, L. (2000) Hazard identification and hazard characterization of *Listeria monocytogenes* in ready-to-eat foods. Joint FAO/WHO expert consultation on risk assessment of microbiological hazards in foods, FAO headquarters, and MRA 00/01. Rome, Italy.

Büla, C.J., Bille, J., Glauser, M.P. (1995) An epidemic of food-borne listeriosis in Western Switzerland: Description of 57 cases involving adults. *Clinical Infectious Diseases* 20, 66-72.

Campbell, A.M. (1996) Bacteriophages. In: *Fields Virology*. pp 587-607. (Editors: Fields, B.N., Knipe, D.M. and Howley, P.M.).New York: Raven Press.

Capita, R., Alonso-Calleja, C., Moreno, C. and García-Fernández, M. C. (2001) Occurrence of *Listeria* species in retail poultry meat and comparison of a cultural/immunoassay for their detection. *International Journal of Food Microbiology* 65, 75-82.

Carlton, R. M. (1999) Phage therapy: past history and future prospects. *Archivum Immunologiae et Therapiae Experimentalis* 47: 267-274.

Carlton, R.M., Noordman, W., Biswas, B., de Meester, E. and Loessner, M.J. (2005) Bacteriophage P100 for control of *Listeria monocytogenes* in foods: genome sequence, bioinformatic analyses, oral toxicity study, and application. *Regulatory Toxicology and Pharmacology* 43, 301–312.

Carrique-Mas, J.J., Hökeberg, I., Andersson, Y., Arneborn, M., Tham, W., Danielsson-Tham, M.L., Osterman, B., Leffler, M., Steen, M., Erikson, E., Hedin, G., Giesecke, J. (2003) Febrile gastroenteritis after eating on-farm manufactured fresh cheese-an outbreak of listeriosis. *Epidemiology and Infection* 130, 79-86

CDC (2007) Outbreak of *Listeria monocytogenes* infections associated with pasteurized milk from a local dairy - Massachusetts. Morbidity and Mortality Weekly Report. 2008; 57:1097-1100.

Chaturongakul, S., Raengpradub, S., Wiedmann M. and Boor, J.K. (2008) Modulation of stress and virulence in *Listeria monocytogenes. Trends in microbiology* 16, 388 -396.

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

Chen, Y., Zhang, W. and Knabel**,** J.S. (2005) Multi-virulence-locus sequence typing clarifies epidemiology of recent Listeriosis outbreaks in the United States. *Journal of Clinical Microbiology* 43, 5291–5298.

Chen, W.T., Hendrickson, R.L., Huang, C.P., Sherman, D., Geng, T., Bhunia, A.K., and Ladisch, M.R. (2005) Mechanistic study of membrane

concentration and recovery of *Listeria monocytogenes*. *Biotechnology and bioengineering* 89, 263-273.

Cherry, W.B., Davis, B.R., Edwards, P.R. and Hogan, R.B. (1954) A simple procedure for the identification of the genus *Salmonella* by means of a specific bacteriophage. *Journal of Laboratory and Clinical Medicine* 44, 51–55.

Chou, C.H., Silva, J.L. and Wang, C. (2006) Prevalence and typing of *Listeria monocytogenes* in raw catfish fillets. *Journal of Food Protection* 69, 815–819.

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

Coffey, B., Mills, S., Coffey, A., McAuliffe, O., and Ross, P. (2010) Phage and their lysins as biocontrol agents for food safety applications. *Annual Review of Food Science and Technology* 1, 449–68.

Coffey, T.M., Nelson, M.B. and Gazzard, G.B. (1989) *Listeria monocytogenes* Meningitis and HIV infected patient. *AIDS*, 3, 614-615.

Cotton, L.N., White, C.H. (1992) *Listeria monocytogenes*, *Yersinia enterocolitica* and *Salmonella* in dairy plant environments. *Journal Dairy Sciences* 75, 51– 57.

Croci, L., Delibato, E., Volpe, G., De Medici, D. and Palleschi, G. (2004) Comparison of PCR, electrochemical enzyme-linked immunosorbent assays, and the standard culture method for detecting *salmonella* in meat products. *Applied and Environmental Microbiology* 70, 1393–1396.

Dauphin, G., Ragimbeau, C. and Malle, P. (2001) Use of PFGE typing for tracing contamination with *Listeria monocytogenes* in three cold-

smoked salmon processing plants. *International Journal of Food Microbiology* 64, 51-61.

Dawson, S.J., Evans, M.R.W., Willby, D., Bardwell, J., Chamberlain, N., and Lewis, D.A. (2006) *Listeria* outbreak associated with sandwich consumption from a local hospital retail shop, United Kingdom. *Euro Surveill* 11, 89-91

De Buyser, M. L., Dufour, B., Maire, M. and Lafarge, V. (2001) Implication of milk and milk products in food-borne diseases in France and in different industrialized countries. *International Journal of Food Microbiology* 67, 1–17.

Deneer, G. H. and Boychuk, I. (1991) Species-Specific Detection of *Listeria monocytogenes* by DNA Amplification. *Applied and Environmental Microbiology* 57, 606-609.

De Siqueira, R.S.C., Dodd, C.E.R. and Rees, C.E.D. (2003) Phage amplification assay as rapid method for *salmonella* detection. *Brazilian Journal of Microbiology* 34, 118-120.

De Siqueira, R.S., Dodd, C.E. and Rees, C.D. (2006) Evaluation of the natural virucidal activity of teas for use in the phage amplification assay. *International Journal of Food Microbiology* 111, 259–262.

Destro, M.T., Leitao, M.F. and Farber, M.J. (1996) Use of molecular typing methods to trace the dissemination of *Listeria monocytogenes* in a shrimp processing plant. *Applied and Environmental Microbiology* 62, 705–711.

Dillon, R. and Patel, T. (1992) *Listeria* in seafoods: a review. *Journal of Food Protection* 55, 1009-1015.

Dmitriev, B.A., Ehlers, S. and Rietschel, E.T. (1999) Layered murein revisited: a fundamentally new concept of bacterial cell wall

structure, biogenesis and function. *Medical Microbiology and Immunology* 187, 173-181.

Donnelly, C.W. (2002) Detection and isolation of *Listeria monocytogenes* from food samples: implications of sublethal injury. *Journal of AOAC International* 85, 495–500.

Donnelly, C.W. and Baigent, G.J. (1986) Method for flow cytometric detection of *Listeria monocytogenes* in milk. *Applied and Environmental Microbiology* 52, 689-695.

Donnelly, C.W. and Nyachuba, D.G. (2007). Conventional methods to detect and isolate *Listeria monocytogenes.* In: *Listeria*, *Listeriosis, and Food Safety*. pp 215–256. (Editors: Ryser, E.T., Marth, E.H. New York: CRC Press.

Drevets, D.A. and Bronze, M.S. (2008) *Listeria monocytogenes*: epidemiology, human disease, and mechanisms of brain invasion. *FEMS Immunology Medical Microbiology* 53, 151-165.

Duffy, G., Walsh, D., Sheridan, J.J., Logue, C.M., Harrington, D., Blair, I.S. and McDowell, D.A. (2001) Comparison of selective and non-selective enrichment media in the detection of *Listeria monocytogenes* from meat containing *Listeria innocua*. *Journal of Applied Microbiology* 90, 994-999.

Eklund, M.W., Poysky, F.R., Paranjpye, R.N., Lashbrook, L.C., Peterson, M.E. and Pelroy, G.A. (1995) Incidence and sources of *Listeria monocytogenes* in cold-smoked fishery products and processing plants. *Journal of Food Protection* 58, 502-508.

Elemam, M.M. (2010) A novel phage-PCR assay for the rapid detection of viable *Listeria monocytogenes* in food within 24 h. *Poster presentation C/P88, ISOPOL XVII, Oporto, Portugal* http://isopol.esb.ucp.pt/docs/abstracts_book2.pdf

Ellis, E.L and Delbrück, M. (1939) The growth of bacteriophage. *Journal of General Physiology* 22, 365–384.

Embarek, P.K. (1994) Presence, detection and growth of *Listeria monocytogenes* in sea foods: a review. *International Journal of Food Microbiology* 23, 17–34.

Ercolini, D., Hill, P.J and Dood, C. (2003) Bacterial community structure and location in Stilton cheese. *Applied and Environmental Microbiology* 69, 3540–3548.

Ericsson, H., Eklow, A., Danielsson-Tham, M.-L., Loncarevic, S., Mentzing, L.-O., Persson, I., Unnerstad, H., Tham, W. (1997a) An outbreak of listeriosis suspected to have been caused by rainbow trout. *Journal of Clinical Microbiology* 35, 2904–2907.

Ericsson, H. and Stålhandske, P. (1997b) PCR detection of *Listeria monocytogenes* in 'gravad' rainbow trout. *International Journal of Food Microbiology* 35, 28 1-285.

European Commission. Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. *Official Journal of the European Union* 2005; **L338**:1-26 (as amended by Regulation (EC) No 1441/2007).

Facinelli, B., Varaldo, E.E., Toni, M., Casolari, C. and Fabio, U. 1989. Ignorance about *Listeria*. *British Medical Journal* 299, 738.

Farber, J.M., Daley, E.M., Mackie, M.T. and Limerick, B. 2000. A small outbreak of listeriosis potentially linked to the consumption of imitation crab meat. *Letters in Applied Microbiology* 31, 100-104.

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

Farber, M.J., Sanders, G.W., Dunfield, S. and Prescott, R. (1989) The effect of various acidulants on the growth of *Listeria monocytogenes*. *Letters in Applied Microbiology* 9, 181-183.

Fernández-Garayzábal, J.F., Suárez, G., Blanco, M.M., Gibello, A., Domínguez, L. (1996) Taxonomic note: a proposal for reviewing the interpretation of the CAMP reaction between *Listeria monocytogenes* and *Rhodococcus equi*. *International Journal of Systematic Bacteriology*, 46, 832-834.

Flekna, G., Stefanic, P., Wagner, M., Smulders, F., Mozina, S. and Hein, I. (2007) Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises EMA/real-time PCR. *Research in Microbiology* 158, 405 – 412.

Fleming, D.W., Cochi, L. S., MacDonald, L. K., Brondum, J., Hayes, S.P., Plikaytis, D.B., Holmes, B.M., Audurier, A., Broome, V.C. and Reingold, L.A. (1985) Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. *The New England Journal* of *Medicine* 312, 404-407.

Garcia, P., Martinez, B., Obeso, J.M., and Rodriguez, A. (2008) Bacteriophages and their application in food safety. *Letters in Applied Microbiology* 47, 479-485.

Gasanov, U., Hughes, D. and Hansbro, M.P. (2005) Methods for the isolation and identification of *Listeria* spp. and *Listeria monocytogenes*: a review. *FEMS Microbiology Reviews* 29, 851–875.

Gaulin, C., Ramsay, D., Ringuette, L., Ismail, J. (2003) First documented outbreak of *Listeria monocytogenes* in Quebec, 2002. *Canada Communicable Disease report* 29, 1-6.

Germini, A., Masola, A., Carnevali, P. and Marchelli, R. (2009) Simultaneous detection of *Escherichia coli O175:H7, Salmonella* spp., and *Listeria monocytogenes* by multiplex PCR. Food Control 20, 733-738.

Gill, J. and Abedon, S.T. (2003) Bacteriophage ecology and plants [WWW document].URL (http://www.apsnet.org/online/feature/phages/).

Gilot, P. and Content, J. (2002) Specific Identification of *Listeria welshimeri* and *Listeria monocytogenes* by PCR Assays targeting a gene encoding a fibronectin-binding protein. *Journal of Clinical Microbiology* 40, 698–703.

Glaser, P., Frangeul, L., Buchrieser, C., Rusniok, C., Amend, A., Baquero, F., Berche, P., Bloecker, H., Brandt, P., Chakraborty, T., Charbit, A., Chetouani, F., Couve, E., de Daruvar, A., Dehoux, P., Domann, E., Dominguez-Bernal, G., Duchaud, E., Durant, L., Dussurget, O., Entian, K.D., Fsihi, H., Garcia-del Portillo, F., Garrido, P., Gautier, L., Goebel, W., Gomez-Lopez, N., Hain, T., Hauf, J., Jackson, D., Jones, L.M., Kaerst, U., Kreft, J., Kuhn, M., Kunst, F., Kurapkat, G., Madueno, E., Maitournam, A., Vicente, J.M., Ng, E., Nedjari, H., Nordsiek, G., Novella, S., de Pablos, B., Perez-Diaz, J.C., Purcell, R., Remmel, B., Rose, M., Schlueter, T., Simoes, N., Tierrez, A., Vazquez- Boland, J.A., Voss, H., Wehland, J. and Cossart, P. (2001) Comparative genomics of *Listeria* species. *Science* 294, 849-852.

Goulet, V., Hedberg, C., Le Monnier, A. and de Valk, H. (2008) Increasing incidence of listeriosis in France and other European countries. *Emerging Infectious Diseases* 14, 734-740.

Goulet, V., Jacquet, C., Vaillant, V., Rebie`re, I., Mouret, E., Lorente, C., Maillot, E., Staıner, F. and Rocourt, J. (1995) Listeriosis from consumption of raw-milk cheese. *Lancet* 345, 1581–1582.

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

Graves, L.M., Helsel, L.O., Steigerwalt, A.G., Morey, R.E., Daneshva,r M.I., Roof, S.E., Orsi, R.H., Fortes ,E.D., Milillo, S.R., den Bakker, H.C., Wiedmann, M., Swaminathan, B., Sauders, B,D. (2010) Listeria marthii sp. nov., isolated from the natural environment, Finger Lakes National Forest. *International Journal of Systematic and Evolutionary Microbiology* 60, 1280–1288.

Gray, M.L. and Killinger, H.A. (1966) *Listeria monocytogenes* and listeric infections. *Microbiology and Molecular Biology Reviews* 30, 309 – 382.

Griffiths, M.W. (1989) *Listeria monocytogenes*: its importance in the dairy industry. *Journal* of the *Science of Food and Agriculture*. 47, 133–158.

Gudmundsdóttir, S., Gudbjörnsdóttir, B., Lauzon, H.L., Einarsson, H., Kristinsson, K.G. and Kristjánsson, M. (2005) Tracing *Listeria monocytogenes* isolates from cold smoked salmon and its processing environment in Iceland using pulsed-field gel electrophoresis. *International Journal of Food Microbiology* 101, 41-51.

Guenther, S., Huwyler, D., Richard, S., Loessner, M. (2009) Virulent bacteriophage for efficient biocontrol of *Listeria monocytogenes* in ready-to-eat foods. *Applied and Environmental Microbiology* 75, 93 – 100.

Guttman, B., Raya, R., and Kutter, E. (2005) Basic phage biology. In: *Bacteriophages: biology and applications.* pp 28. (Editors: Kutter, E. and Sulakvelidze, A.) CRC Press, Boca Raton, FL.

Hagens, S. and Loessner, J. M. (2007) Bacteriophages of *Listeria*. I pp 265-279. Institute of Food Science and Nutrition, EPI Food safety, ETH Zurich, Switzerland.

Hayes, W. (1968) The Genetics of Bacteria and their Viruses, Second Edtion. Oxford & Edinburgh: United Kingdom. Blackwell Scientific Publications.

Health Canada. (2010) Policy on *Listeria monocytogenes* in ready-to-eat foods, Version 001. Health Canada, Health Products and Food Branch, Food Directorate.

Health Canada. (2011) Policy on Listeria monocytogenes in ready-to-eat. (http://www.hc-sc.gc.ca/fn-an/legislation/pol/policy-listeria_ monocytogenes_2011-eng.php).

Heinitz, M.L. and Johnson, J.M. (1998) The incidence of *Listeria* spp., *Salmonella* spp., and *Clostridium botulinum* in smoked fish and shellfish. *Journal of Food Protection* 61, 318-323.

Helvoort, T.V. (1992) Bacteriological and physiological research styles in the early controversy on the nature of the bacteriophage phenomenon. *Medical History* 36, 243–270.

Hicks, S.J. and Lund, B.M. (1991) The survival of *Listeria monocytogenes* in cottage cheese. *Journal of Applied Bacteriology*70, 308-314.

Hird, D.W and Geaigeorgias, C. (1990) Listeriosis in food animals: clinical signs and livestock as a potential source of direct (non-foodborne) infection for humans. In: *Foodborne listeriosis*. pp 31-40. (Editors: Miller, L.A., Smith, L.A. and Somkuti, A.G.) Amsterdam, Netherlands: Elsevier Science Publishers.

Hirsh, C.D., and Martin, D.L. (1983) Rapid Detection of *Salmonella* spp. by Using Felix-O1 Bacteriophage and High-Performance Liquid

Chromatography. *Applied and Environmental Microbiology* 45, 260-264.

Hitchins, A.D. (1996) Assessment of alimentary exposure to *Listeria monocytogenes*. *International Journal of Food Microbiology* 30, 71-85.

Hitchins, D. and Jinneman, K. (2011) Detection and enumeration of *Listeria monocytogenes* in Foods. FDA. Bacteriological Analytical Manual. Chapter 10.

Holko, J., Urbanova, M., Kantikova, K., Pastorova, A. and Kmee, V. (2002) PCR Detection of *Listeria monocytogenes* in Milk and Milk Products and Differentiation of Suspect Isolates. *ACTA Vet. Brno*. 71, 125–131.

Holtje, J.V. (1998) Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*. *Microbiology and Molecular Biology Reviews* 62, 181-203.

Hsih, H.Y. and Tsen, H.Y. (2001) Combination of immunomagnetic separation and polymerase chain reaction for the simultaneous detection of *Listeria monocytogenes* and Salmonella spp. in food samples. *Journal of Food Protection* 64, 1744–1750.

Hudson, J.A., Billington, C. Carey-Smith, G. and Greening, G. (2005) Bacteriophages as biocontrol agents in food. *Journal of Food Protection* 68, 426–437.

Hudson, J.A., Lake, R.J., Savill, M.G., Scholes, P. and McCormick, R.E. (2001) Rapid detection of *Listeria monocytogenes* in ham samples using immunomagnetic separation followed by polymerase chain reaction. *Journal of Applied Microbiology* 90, 614–621.

Hwang, C.A. and Marmer, S.B. (2007) Growth of *Listeria monocytogenes* in egg salad and pasta salad formulated with mayonnaise of various pH and stored at refrigerated and abuse temperatures. *Food Microbiology* 24, 211 – 218.

ISO (1996) Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *Listeria monocytogenes*. Part 1: Detection method. ISO method 11290-1, 1st ed. International Organization for Standardization. Genève, Switzerland.

Ivanek, R., Gröhn, T.Y. and Wiedmann, M. (2006) *Listeria monocytogenes* in multiple Habitats and host populations: Review of available data for mathematical modeling. *Foodborne Pathogens and Disease* 3, 319-336.

Jadhav, S., Bhave, M. and Palombo, A.E. (2012) Methods used for the detection and subtyping of *Listeria monocytogenes*. *Journal of Microbiological Methods* 88, 327–341.

Janda, M.J. and Abbott, L.S. (2007) 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, Perils, and Pitfalls. *Journal of Clinical Microbiology* 45, 2761–2764.

Janzten, M.M., Navas, J., Corujo, A., Moreno, R., López, V. and Martínez-Suárez. V.J. (2006) Specific detection of *Listeria monocytogenes* in foods using commercial methods: from chromogenic media to real-time PCR. *Spanish Journal of Agricultural Research* 4, 235 – 247.

Jassim, S.A.A. and Mazen, A.N. (2007) In vitro Evaluation of the Antiviral Activity of an extract of date palm (Phoenix dactylifera L.) pits on a *Pseudomonas Evidence-Based Complementary and Alternative Medicine* 7, 1- 6.

Jemmi, T. and Keusch, A. 1994. Occurence of *Listeria monocytogenes* in freshwater fish farms and fish-smoking plants. *Food Microbiol* 11, 309-316.

Jemmi, T. and Stephan, R. (2006) *Listeria monocytogenes*: food-borne pathogen and hygiene indicator. *Revue Scientifique et Technique-Office Iinternational des Epizooties* 25, 571-580.

Jeršek, B., Majstorović, T., Klun, N. and Možina, S. 2005. Impact of enrichment medium on PCR-based detection of *Listeria monocytogenes* in food. *Acta Agriculturae Slovenica* 85, 15-23.

Jeyaletchumi, P., Tunung, R., Margaret, S.P., Son, R., Farinazleen, M.G., Cheah, Y.K. (2010) Detection of *Listeria monocytogenes* in foods. *International Food Research Journal* 17, 1-11.

Johansson, T. (1998) Enhanced detection and enumeration of *Listeria monocytogenes* from foodstuffs and food-processing environments. *International Journal of Food Microbiology* 40, 77–85.

Johansson, T., Rantalaa, L., Palmub, L. and Honkanen-Buzalskia, T. (1999) Occurrence and typing of *Listeria monocytogenes* strains in retail vacuum-packed fish products and in a production plant. *International Journal of Food Microbiology* 47,111–119.

Johnson, J.L., Doyle, P.M., and Cassens, G.R. (1988) Survival of *Listeria monocytogenes* on ground beef. *International Journal of Food Microbiology* 6, 243-247.

Juntila, J.R., Niemela, I.S., and Hirn, J. (1988) Minimum growth temperatures of *Listeria monocytogenes* and non haemolytic *Listeria*. *Journal of Applied Bacteriology* 65, 321 – 327.

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

Kim, S., Park, M., Kim, J., Chuong, D.P., Lee, Y., Yoon, B., Hwang, K., and Lim, Y. (2005) Development of a sandwich ELISA for the detection of *Listeria* spp. Using specific flagella antibodies. *Journal of Veterinary Science* 6, 41–46.

Kim, J.W., Siletzky, M.R., and Kathariou, S. (2008) Host ranges of *Listeria*-specific bacteriophages from the turkey processing plant environment in the United States. *Applied and Environmental Microbiology* 74, 6623–6630.

Klausner, R.B., and Donnelly, C.W. (1991) Environmental sources of *Listeria* and *Yersinia* in Vermont dairy plants. *Journal of Food Protection* 54, 607–611.

Knabel, S.J. (2002) Optimized, one-step, recovery-enrichment broth for enhanced detection of *Listeria monocytogenes* in pasteurized milk and hot dogs. *Journal of AOAC International* 85, 501-504.

Kumar, R. (2011) Modern trends to investigate food borne Listeriosis. *Journal of Food Technology* 9l, 9-17.

Kuroda, Y. and Y. Hara (1999) Antimutagenic and anticarcinogenic activity of tea polyphenols. *Mutation Research/Reviews in Mutation Research* 436, 69-97.

Kutter, E., and Sulakvelidze, A. (2005) Introduction. In: Bacteriophages: *biology and applications.* pp 1 (Editors: Kutter, E. and Sulakvelidze, A.) CRC Press, Boca Raton, FL.

Lammerding, M.A. and Doyle, P.M. (1989) Evaluation of enrichment procedures for recovering *Listeria monocytogenes* from dairy products. *International Journal of Food Microbiology* 9, 249–268.

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

Lawrence, L.M. and Gilmour, A. (1994) Incidence of *Listeria* spp., *Listeria monocytogenes* in a poultry processing environment and in poultry

products and their rapid confirmation by multiplex PCR. *Applied and Environmental Microbiology* 60, 4600–4604.

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

Leclercq, A., Clermon,t D., Bizet, C., Grimont, P.A., Le Flèche-Matéos, A., Roche, S.M., Buchrieser, C., Cadet-Daniel, V., Le Monnier, A., Lecuit, M., Allerberger, F. (2010) Listeria rocourtiae sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 60, 2210–2214.

Lennon, D., Lewis, B., Mantell, C., Becroft, D., Dove, B., Farmer, K., Tonkin, S., Yeates, N., Stamp, R. and Mickleson, K. 1984. Epidemic perinatal listeriosis. *The Pediatric Infectious Disease Journal* 3, 30-34.

Leverentz, B., Conway, W.S., Camp, M.J., Janisiewicz, W.J., Abuladze, T., Yang, M., Saftner, R. and Sulakvelidze, A. (2003) Biocontrol of *Listeria monocytogenes* on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. *Applied and Environmental Microbiology* 69, 4519–4526.

Leverentz, B., Conway, W.S., Janisiewicz, W. and Camp, M.J. (2004) Optimizing concentration and timing of a phage spray application to reduce *Listeria monocytogenes* on honeydew melon tissue. *Journal of Food Protection* 67, 1682– 1686.

Li, X., Boudjellab, N. and Zhao, X. (2000) Combined PCR and slot blot assay for detection of Salmonella and Listeria monocytogenes. *Internatiolnal Journal of Food Microbiology* 56, 167–177.

Lianou, A. and Sofos, J.N. (2007) A review of the incidence and transmission of *Listeria monocytogenes* in ready-to-eat products in retail and food service environments. *Journal of Food Protection* 70, 2172-2198.

Lillehaug, D. (1997) An improved plaque assay for poor plaque-producing temperate lactococcal bacteriophages. *Journal of Applied Microbiology* 83, 85-90.

Lindberg, A. (1973) Bacteriophage receptors. *Annual Review of Microbiology* 27, 205-241.

Little, C.L., Sagoo, S.K., Gillespie, I.A., Grant, k., Mclauchlin, J. (2009) Prevalence and level of *Listeria monocytogenes* and other *Listeria* species in selected retail ready-to-eat foods in the United Kingdom. *Journal of food Protection* 72, 1869-1877.

Liu, D., Ainsworth, A.J., Austin, F.W. and Lawrence, M.L. (2004) Identification of a gene encoding a putative phosphotransferase system enzyme IIBC in *Listeria welshimeri* and its application for diagnostic PCR. *Letters in Applied Microbiology* 38, 151–157.

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

Loessner, M.J., Estela, L. A., Zink, R. and Scherer, S. (1994) Taxonomical classification of 20 newly isolated *Listeria* bacteriophages by electron microscopy and protein analysis. *Intervirology* 37, 31–35.

Loessner, J.M., Krause, L.B., Henle, T. and Scherer, S. (1994) Structural proteins and DNA characteristics of 14 *Listeria* typing bacteriophages. *The Journal of General Virology* 75, 701-710.

Loessner, J.M., Inman, R.B., Lauer, P., Calendar, R. (2000) Complete nucleotide sequence, molecular analysis and genome structure of bacteriophage A118 of *Listeria monocytogenes*: implications for phage evolution. *Molecular Microbiology* 35, 324-40.

Loessner, J.M., Rees, C.E., Stewart, S.G., and Scherer, S. (1996) Construction of luciferase reporter bacteriophage A511: luxAB for rapid and

sensitive detection of viable *Listeria* cells. *Applied and Environmental Microbiology* 62, 1133–1140.

Loessner, J.M., Rudolf, M. and Scherer, S. (1997) Evaluation of luciferase reporter bacteriophage A511: luxab for detection of *Listeria monocytogenes* in contaminated foods. *Applied and Environmental Microbiology* 63, 2961–2965.

Loessner, J.M., and Scherer, S. (1995) Organization and transcriptional analysis of the Listeria phage A511 late gene region comprising the major capsid and tail sheath protein genes *cps* and *tsh*. *Journal of Bacteriology* 177, 6601–6609.

Longhi, C., Maffeo, A., Penta, M., Seganti, L. and Conte, M. (2003) Detection of *Listeria monocytogenes* in Italian-style soft cheeses. *Journal of Applied Microbiology* 94, 879-885.

Łos, J.M., Golec, P., Grzyn, W.G., Grzyn, A. W. and Łos, M. (2008) Simple method for plating *Escherichia coli* bacteriophages forming very small plaques or no plaques under standard conditions. *Applied and Environmental Microbiology* 74, 5113–5120.

Los, M., Golec, P., Los, J. M., Weglewska-Jurkiewicz, A., Czyz, A., Wegrzyn, A., Wegrzyn, G., and Neubauer, P. (2007) Effective inhibition of lytic development of bacteriophages λ, P1 and T4 by starvation of their host, Escherichia coli." *BMC Biotechnology* 7, 13.

Lunden, J., Tolvanen, R., and Korkeala, H. (2004) Human listeriosis outbreaks linked to dairy products in Europe. *Journal of Dairy Science* 87, 6–12.

Lundén, J.M., Miettinen, M.K., Autio, T.J. and Korkeala, H.J. (2000) Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact times. *Journal of Food Protection* 63, 1204-1207.

Lyytikainen, O., Autio, T., Maijala, R., Ruutu, P., Honkanen-Buzalski, T., Miettinen, M., Hatakka, M., Mikkola, J., Anttila, V., Johansson, T., Rantala, L., Aalto, T., Korkeala, H., and Siitonen, A (2000) An Outbreak of *Listeria monocytogenes* serotype 3a infections from butterin Finland. *The Journal of Infectious Diseases* 181, 1838–1841.

Makhtar H, Hatiyar, S., Agarwal, P. (1994) Green tea and skin anticarcinogenic effect. *Journal of Investigative Dermatology* 102, 3-7

Makino, I.S., Kawamoto, K., Takeshi, K., Okada, Y., Yamasaki, M., Yamamoto, S. (2005) An outbreak of food-borne Listeriosis due to cheese in Japan, during 2001. *International Journal of Food Microbiology* 104, 189-196.

Maloy, S.R., Cronan, J.E., and Freifelder, D. (1994) Microbial genetics. pp 121-122. second edition. Boston, MA: Jones and Bartlett Publishers, Inc.

Mandal, P.K., Biswas, A.K., Choi, K. and Pal, U.K. (2011) Methods for rapid detection of foodborne pathogens: An Overview. *American Journal of Food Technology* 6, 87-102.

Maniloff, J. and Ackermann, H.W. (1998) Taxonomy of bacterial viruses: establishment of tailed virus genera and the order Caudovirales. *Archives of Virology* 143, 2051-2063.

Manzano, M., Cocolin, L., Ferroni, P., Gasparini, V., Varduzzi, D., Cantoni, C. and Comi, G. (1996) Identification of *Listeria* species by a semi-nested polymerase chain reaction. *Research in Microbiology*. 147, 637-640.

Marei, A.M., El-Behedy, M.E., Mohtady, A.H. and Afify, A.F. (2003) Evaluation of a rapid bacteriophage-based method for the detection of *Mycobacterium tuberculosis* in clinical samples. *Journal of Medical Microbiology* 52, 331–335.

Markoulatos, P., Siafakas, N. and Moncany, M. (2002) Multiplex polymerase chain reaction: A practical approach. *Journal of Clinical Laboratory Analysis* 16, 47–51.

Martín, B., Jofré, A., Garriga, M., Hugas, M. and Aymerich, T (2004) Quantification of *Listeria monocytogenes* in fermented sausages by MPN-PCR method. *Letters in Applied Microbiology* 39, 290-295.

Mathakiya, R.A., Roy, A. and Nayak, J.B. (2011) Characterization of *Listeria monocytogenes* isolates by CAMP test. *Veterinary World* 4, 301-303.

McCarthy, S.A. (1990) *Listeria* in the environment. *In: Foodborne listeriosis.* pp 25-29. (Editors: Miller, A. J., Smith, J.L. and Somkuti, G.A). Society for Industrial Microbiology. Elsevier Science Publishing, Inc., New York.

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

McKinstry, M. and Edgar, R. (2005) Phages: Their Role in Bacterial Pathogenesis and Biotechnology. pp. 430–440. (Editors: Matthew, K.W., Friedman, D.I. and Adhya, S.L.) American Society Microbiology, Washington, DC.

McGowan, A.P., Marshall, R.J. and Reeves, D.S. (1989) Evaluation of API 20 strep system for identifying *Listeria* species. *Journal of Clinical Pathology*, 42, 548-550.

McLauchlina, J., Mitchellb, R.T., Smerdonc, W.J. and Jewelld, K. (2004) *Listeria monocytogenes* and listeriosis: a review of hazard characterization for use in microbiological risk assessment of foods. *International Journal of Food Microbiology* 92, 15– 33.

McNerney, R., Kambashi, B.S. Kinkese, J., Tembwe, R. and Godfrey-Faussett, P. (2004) Development of a bacteriophage phage replication assay for

diagnosis of pulmonary tuberculosis. *Journal of Clinical Microbiology*, 42, 2115–2120.

McNerney, R. Wilson, S.M., Sidhu, A.M., Harley, V.S., Suwaidi, Z., Nye, P.M., Parish, T. and Stoker, N.G. (1998) Inactivation of Mycobacteriophage D29 using ferrous ammonium sulphate as a tool for the detection of viable *Mycobacterium smegmatis* and M. tuberculosis. *Research in Microbiology* 149:487–495.

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

Menendez, S., Godinez, M.R., Rodriguez-Otero, J.L. and Centeno, J.A. (1997) Removal of *Listeria* spp. in a cheese factory. *Journal of Food Safety* 17,133–139.

Miettinen, M.K., Siitonen, A., Heiskanen, P., Haajanen, H., Björkroth, K.J. and Korkeala, H.J. 1999. Molecular epidemiology of an outbreak of febrile gastroenteritis caused by *Listeria monocytogenes* in cold-smoked rainbow trout. *Journal of Clinical Microbiology* 37, 2358-2360.

Molineux, I.J. (2006) Fifty-three years since Hershey and Chase; much ado about pressure but which pressure is it? *Virology* 344, 221-229.

Mook, P., Grant, K.A., Little, C.L., Kafatos, G., Gillespie, A. (2010) Emergence of pregnancy-related listeriosis amongst ethnic minorities in England and Wales. Euro Surveill. 15 (27). pii= 19610: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19610.

Muller, A.A., Schmid, M.W., Meyer, O. and Meussdoerffer, G. (2010) *Listeria seeligeri* isolates from food processing environments form two

phylogenetic lineages. *Applied and Environmental Microbiology* 76, 3044–3047.

Nakamura, H., Hatanaka, M., Ochi, K., Nagao, M., Ogasawara, J., Hase, A., Kitase, T., Haruki, K. and Nishikawa, Y. (2004) *Listeria monocytogenes* isolated from cold-smoked fish products in Osaka city, Japan. *International Journal of Food Microbiology* 94, 323-328.

NCFA. (1999) *Listeria monocytogenes* detection in foods. 136. Nordic Committee on Food Analysis. 2nd ed. http://www.nmkl.org/.

Niederhauser, C., Candrin, U., Hofelein, C., Jermini, M., Buhler, P.H. and Luthy, J. (1992) Use of polymerase chain reaction for detection of *Listeria monocytogenes* in food. *Applied Environmental Microbiology* 58, 1564 – 1568.

Nørrung, B., Andersen, J.K. and Schlund, J. (1999) Incidence and control of *Listeria monocytogenes* in foods in Denmark. *International Journal of Food Microbiology* 53, 195-203.

Norton, D.M., McCamey, M.A., Gall, K., Scarlett, J.M., Boor, K. J. and Wiedmann, M. (2001a) Molecular studies on the ecology of *Listeria monocytogenes* in the smoked fish processing industry. *Applied and Environmental Microbiology* 67, 198-205.

Norton, D., Scarlett, J., Horton, K., Sue, D., Thimothe. J., Boor, K. and Wiedmann, M. (2001b) Characterization and pathogenic potential of *Listeria monocytogenes* isolates from the smoked fish industry. *Applied and Environmental Microbiology* 67, 646-653.

Nwaiwu, O. (2011) The effect of growth conditions on the surface properties of *Listeria monocytogenes*, PhD Thesis, University of Nottingham.

O'Grady, J., Ruttledge, M., Sedano-Balba, S. Smith, J.T., Majella, M. (2009) Rapid detection of *Listeria monocytogenes* in food using culture enrichment combined with real-time PCR. *Food Microbiology* 26, 4–7.

Oladepo, D.K., Candlish, A.A.G. and Stimson, W.H. (1992) Detection of *Listeria monocytogenes* using polyclonal antibody. *Letters* in *Applied Microbiology* 14, 26-29.

Palumbo, D.J., Borucki, K.M., Mandrell, E.R. and Gorski, L. (2003) Serotyping of *Listeria monocytogenes* by enzyme-linked immunosorbent assay and identification of mixed-serotype cultures by colony immunoblotting. *Journal of Clinical Microbiology* 41**,** 564-571**.**

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

Park, D.J., Drobniewski, F., Meyer, A. and Wilson, S.M. (2003) Use of a phage-based assay for phenotypic detection of *Mycobacteria* directly from sputum. *Journal of Clinical Microbiology* 41, 680-688.

Parish, M.E., and Higgins, P.D. (1989) Survival of *Listeria monocytogenes* in low pH model broth systems. *Journal Food Protection* 52, 144-147

Patel, R. and Paya, C.V (1997) Infections in solid-organ transplant recipients. *Clinical Microbiology Reviews* 10, 86–124.

Perrin**,** M., Bemer**,** .M. and Delamare, C. (2003) Fatal case of *Listeria innocua* bacteremia. *Journal of Clinical Microbiology* 41, 5308 - 5309.

Peterson, B.W., Sharma, P.K., van der Mei, H.C., Busscher H.J. (2012) Bacterial cell surface damage due to centrifugal compaction. *Applied and Environmental Microbiology* 78, 120-125.

Pirie, J. H. H. 1940. The genus Listerella Pirie. Science 91: 383.

Poltronieri, P., De Blasi, M.D. and D'Urso, O.F. (2009) Detection of *Listeria monocytogenes* through real-time PCR and biosensor methods. *Plant Soil and Environment* 55, 363–369

Premaratne, R. J., Lin, W.J. and Johnson, E.A. (1991) Development of an improved chemically defined minimal medium for *Listeria monocytogenes*. *Applied Environmental Microbiology* 57, 3046–3048.

Pritchard, T.J., Flanders, K.J. and. Donnelly, C.W (1995) Comparison of the incidence of *Listeria* on equipment versus environmental sites within dairy processing plants. *International Journal of Food Microbiology* 26, 375- 384.

Rakhuba, V.D. Kolomiets, I.E., Szwajcer, E., and Novik, I.G. (2010) Bacteriophage receptors, mechanisms of phage adsorption and penetration into host cell. *Polish Journal of Microbiology* 59, 145-155.

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

Rebagliati, V., Philippi, R., Rossi, M. and Troncoso, A., (2009) Prevention of food-borne listeriosis. *India Journal of Pathology and Microbiology* 52, 145-149.

Rees, C.E.D and Dodd, C.E.R (2006) Phage for rapid detection and control of bacterial pathogens in food. *Advances in Applied Microbiology* 59,159–86.

Rees, C.E.D. and Botaris, G. (2012) "The use of phage for detection, antibiotic sensitivity testing and enumeration" in understanding Tuberculosis - Global experiences and innovative approaches to the diagnosis, Intech. P-J Cardona (ed) Chapter 14, pp 293-306.

Rees, J.C. and Voorhees, K.J. (2005) Simultaneous detection of two bacterial pathogens using bacteriophage amplification coupled with matrix-

assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry* 19, 2757–2761.

Riedo, F.X., Pinner, R.W., de Lourdes Tosca, M., Cartter, M.L., Graves, L.M., Reeves, M.W., Weaver, R.E., Plikaytis, B.D. and Broome, C.V. (1994) A point-source foodborne listeriosis outbreak: documented incubation period and possible mild illness. Infectious Disease *Journal* 170, 693-696.

Rip, D. and Gouws, P.A. (2009) Development of an internal amplification control using multiplex PCR for the detection of *Listeria monocytogenes* in food products. *Food Analytical Methods* 2, 190–196.

Rittenburg, J.H. (1990) Fundamentals of immunoassay. In Development and Application of lmmunoassay for Food Anafysis. R.H. Rittenburg (Ed.). Elsevier Science Publishers Ltd., New York, NY. pp. 29-58.

Roberts, J.A. and Wiedmann, M. (2003) Pathogen, host and environmental factors contributing to the pathogenesis of listeriosis. *Cellular and Molecular Life Sciences* 60, 904–918.

Rocourt, J. (1986) Bacteriophages and bacteriocins of the genus *Listeria*. *Zentralbl. Bakteriol Microbial Hyg* 1, 12-28.

Rodríguez-Lázaro, D., Jofré, A., Aymerich, T., Garriga, M. and Pla, M. (2005) Rapid quantitative detection of *Listeria monocytogenes* in salmon products: evaluation of pre- real-time PCR strategies. *Journal of Food Protection* 68, 1467-1471.

Rørvik, L.M. (2000) *Listeria monocytogenes* in the smoked salmon industry. *International Journal of Food Microbiology* 62, 183-190.

Rørvik, L.M., Caugant, D.A. and Yndestad, M. (1995) Contamination pattern of *Listeria monocytogenes* and other *Listeria* spp. in a salmon slaughterhouse and smoked salmon processing plant. *International Journal of Food Microbiology* 25, 19-27.

Rørvik, L.M., Skjerve, E., Knudsen, B.R. and Yndestad, M., (1997) Risk factors for contamination of smoked salmon with *Listeria monocytogenes* during processing. *International Journal of Food Microbiology* 37, 215-219.

Rossen, L., Holmstrom, K., Olsen, E.J. and Rasmussen, F.O. (1991) A rapid polymerase chain reaction (PCR)-based assay for the identification of *Listeria monocytogenes* in food samples. *International Journal of Food Microbiology* 14, 145–151.

Ruban, W.S., Sharada, R. and Banday, G.T. (2001) Physical Methods of Separation and Concentration of Microbes in Food: An Aid for Rapid Detection 9, 106-111.

Ryser, E.T. and Marth, H.E. (1999) *Listeria*, listeriosis, and food safety. In: *Listeriosis in animals*. pp 39-57. Second Edition. (Editor: Wesely, A.) Marcel Dekker, Inc. New York, USA.

Sakanaka, S., Kim, M., Taniguchi, M. and Yamamoto, T. (1989) Antibacterial substances in Japanese green tea extract against *Streptococcus mutans* a cariogenic bacterium. *Agricultural and Biological Chemistry* 53, 2307-2311.

Schuchat, A., Swaminathan, B. and Broome, C. V. (1991) Epidemiology of human disease. *Clinical Microbiology Reviews* 4, 169-183.

Schmelcher, M and Loessner, J.M. (2008) Bacteriophage: Powerful Tools for the Detection of Bacterial Pathogens. In: *Principles of Bacterial Detection: Biosensors, Recognition Receptors and Microsystems*, pp.731-754 (Editor: Zourob, M.) Springer Science + Business Media, LLC.

Seeliger, H.P.R. and Jones, D. (1986) The genus *Listeria*. (Editors: Sneath, P.H.A., Mair, N S., Sharpe, M.E. and Holt, J.G.) Bergey's manual of systemic bacteriology. pp 1235-1245. Baltimore: Williams &Wilkins.

Sillankorva, S., Pleteneva, E., Shaburova, O., Santos, S., Carvalho, C., Azeredo, J., and Krylov, V. (2010) *Salmonella* Enteritidis bacteriophage candidates for phage therapy of poultry. *Journal of Applied Microbiology* 108, 1175-1186.

Simon, M.C., Gray, D.I. and Cook, N. (1996) DNA extraction and PCR methods for the detection of *Listeria monocytogenes* in cold-smoked salmon. *Applied and Environmental Microbiology* 662, 822-824.

Somers, E.B. and Wong, A.C. (2004) Efficacy of two cleaning and sanitizing combinations on *Listeria monocytogenes* biofilms formed at low temperature on a variety of materials in the presence of ready-to-eat meat residue. *Journal of Food Protection* 67, 2218-2229.

Somer, L. and Kashi, Y. (2003) A PCR method based on 16S rRNA sequence for simultaneous detection of the genus *Listeria* and the species *Listeria monocytogenes* in food products. *Journal of Food Protection* 66, 1658 - 65.

Sorinano, J.M., Rico, H., Molto, J.C., and Manes, J. (2001) *Listeria* species in raw and ready-to-eat foods from restaurants. *Journal of Food Protection* 64, 551-553.

Stanley, E.C., Richard, J.M., Rebecca, J.S., Sarah M.G., Michael, R.B., McGowan, M. and Rees, C.E. (2007) Development of a new combined rapid method using phage and PCR for detection and identification of viable *Mycobacterium paratuberculosis* bacteria within 48 hours. *Applied and Environmental Microbiology* 73, 1851–1857.

Stessl, B., Luf, W., Wagner, M., and Schoder, D. (2009) Performance testing of six chromogenic ALOA-type media for the detection of *Listeria monocytogenes*. Journal *of Applied Microbiology* 106, 651-659.

Stevens, K.A. and Jaykus, L.A. (2004) Bacterial separation and concentration from complex sample matrices: A review. *Critical Reviews in Microbiology* 30, 7-24.

Stewart, G.S.A.B., Denyer, S.P. and Jassim, S.A.A. (1995) Antiviral and antifungal composition and method. PCT patent WO 95/22254.

Stewart, G.S.A.B., Jassim, S.A.A., Denyer, S.P., Rees, C.E.D., Park, S. and Rostas-Mulligan, K. (1992) Methods for rapid microbial detection. PCT patent WO 92/02633.

Stewart, G.S.A., Jassim, S. Denyer, S.P., Newby, P., Linley, K. and Dhir, V.K. (1998) The specific and sensitive detection of bacterial pathogens within 4 h using bacteriophage amplification. *Journal of Applied Microbiology* 84, 777 – 783.

Strauch, E., Hammerl, J., and Hertwig, S. (2007) Bacteriophages: New Tools for Safer Food? *Journal fur Verbraucherschutz und Lebensmittelsicherheit* 2, 138-143.

Supanivatin, P. Kosonpisit, K. Liamkaew, R., Saeteawand, N. and Thipayarat, A. (2012) Inhibitory effects of Listeria selective enrichment media on growth characteristics of *L. ivanovii*. *Procedia Engineering* 32, 112–118.

Schuchat, A., B. Swaminathan and C. V. Broome. (1991) Epidemiology of human disease. *Clinical Microbiology Review* 4, 169-183.

Swaminathan, B. and Gerner-Smidt, P. (2007) The epidemiology of human listeriosis. *Microbes and Infection* 9, 1236-1243.

Thimothe, J., Walker, J., Suvanich, V., Gall, K.L., Moody, M.W., Wiedmann, M. (2002) Detection of *Listeria* in crawfish processing plants and in raw, whole crawfish and processed crawfish (*Procambarus* spp.). *Journal of Food Protection* 65, 1735–1739.

Thomas, E.J., King, R.K., Burchal, J, and Gannon, P.V. (1991) Sensitive and specific detection of *Listeria monocytogenes* in milk and ground beef with the polymerase chain reaction. *Applied and Environmental Microbiology* 57, 2576–2580.

Todd, E.C.D. and Notermans, S. (2011) Surveillance of listeriosis and its causative pathogen, *Listeria monocytogenes*. *Food Control* 22, 1484 – 1490.

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

Trivett, L.T., Meyer, A.E. (1971) Citrate cycle and related metabolism of *Listeria monocytogenes*. *Journal of Bacteriology* 107, 770-779**.**

Urbanova, H. J., Kantikova, M., Pastorova, K. and Kmee, V. (2002) *ACTA VET. BRNO* 71, 125–131

Vaz-Velho, M., Duartea, G. and Gibbs, P. (2001) Comparison of two pre-enrichments broths for recovering *Listeria* spp. from salmon (*Salmo salar*) and salmon-trout (*Oncorhynchus mykiss*). *Food Control* 12, 357–359.

Voller, A., Bartlett, A. and Bidwell, D.E (1978) Enzyme immunoassays with special reference to ELISA techniques. *Journal Clinical Pathology* 31, 507-520.

Waak, E., Tham, W., and Danielsson-Tham, M.L. (1999) Comparison of the ISO and IDF methods for detection of *Listeria monocytogenes* in blue veined cheese. *International Dairy Journal* 9, 149–155.

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

Walker, S.J. and Stringer, F.M. (1987) Growth of *Listeria monocytogenes* and *Aeromonas hydrophila* at chill temperatures. *Journal and Applied of Bacteriology* 63, 20-26.

Wang, C. and Hong, C. (1999) A rapid PCR-based hybridization assay for the detection of *Listeria monocytogenes* in channel catfish. *Food Microbiology* 16, 29 1-297.

Wang, I.N., Smith, D.L., and Young, R. (2000) Holins: the protein clocks of bacteriophage infections. *Annual Reviews in Microbiology* 54**,** 799-825.

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

Warburton, D.W., Farber, J.M., Armstrong, A., Caldeira, R., Tiwari, N.P., Babiuk, T., LaCasse, P., and Reed, S. (1991) A Canadian comparative study of modified versions of the "FDA" and "USDA" methods for the detection of *Listeria monocytogenes. Journal of Food Protection* 54, 669–676.

Wemekamp-Kamphuis, H.H., Karatzas, K.A., Wouters, A.J. and Abee, T (2002) Enhanced levels of cold shock proteins in *Listeria monocytogenes* LO28 upon exposure to low temperature and high hydrostatic pressure. *Applied and Environmental Microbiology* 68, 456-463.

Wesley, I.V., Harmon, K.M., Dickson, J.S. and Schwartz, A.R. (2002) Application of a multiplex polymerase chain reaction assay for the simultaneous confirmation of *Listeria monocytogenes* and other *Listeria* species in turkey sample surveillance. *Journal of Food Protection* 65, 780–785.

Yang, H., Yang, L., Wimbrow, N. A., Jiang, X. and Sun, Y. (2007) Rapid detection of *Listeria monocytogenes* by nanoparticle-based immunomagnetic separation and real-time PCR. *International Journal of Food Microbiology* 118, 132–138.

Young, I., Wang, I., and Roof, W.D. (2000) Phages will out: strategies of host cell lysis. *Trends in Microbiology* 8**,** 120-128.

Zameer, F and Gopal, S (2010) Impact of hydrogen peroxide on growth and survival of *Listeria monocytogenes* biofilms. *E-Journal of Chemistry* 7**,** 1008-1012.

Zink, R. and Loessner, J. M. (1992) Classification of virulent and temperate bacteriophages of Listeria spp. on the basis of morphology and protein analysis. *Applied and Environmental Microbiology* 58, 296-302.

Zottola, E.A. (1994) Microbial attachment and biofilm formation: a new problem for the food industry? *Food Technology* 48, 107-114.

Zunabovic, M., Domig, J.K. and Kneifel, w. (2011) Practical relevance of methodologies for detecting and tracing of *Listeria monocytogenes* in ready-to-eat foods and manufacture environments. A review. *Food Science and Technology* 44, 351-362.

APPENDICES

APPENDIX 1

COMPOSITION OF MEDIA AND REAGENT USED

Brain Heart Infusion Broth

Brain-heart infusion solid	17.5 g
D (+) glucose	2.0 g
NaCl	5.0 g
Na_2HP0_4	2.5 g
Tryptose	10 g
pH	7.4 (± 0.2)
Distilled water	1 liter

Trypticase soy agar

Tryptone	15 g
Soytone - enzymatic digest of soybean	5.0 g
NaCl	5.0 g
pH	7.4 (± 0.2)
Agar	15 g
Distilled water	1 liter

Fraser medium

Peptone from casein	5.0 g
Peptone from meat	5.0 g
Yeast-extract	5.0 g
Meat-extract	5.0 g
NaCl	20.0 g
Na_2HPO_4	12.0 g
KH_2P0_4	1.35 g
Esculine	1.0 g
Lithium chloride	3.0 g
Nalidixic acid	0.02 g
Distilled water	1 liter

Oxford agar plates

Columbia-agar-base	39.0 g
Esculine	1.0 g
Iron (III) ammonium citrate	0.5 g
Lithium chloride	15.0 g
pH	7.0 (± 0.2)
Distilled water	1 liter

Listeria Selective Supplement (Oxford Formulation SR140)

Vial Contents

SR 140E to supplement 500 of Listeria agar medium

Acriflavine	2.5 mg
Cefotatan	1.0 mg
Colistin sulphate	10 mg
Cycloheximide	200 mg
Fosfomycin	5.0 mg

PALCAM Selective Agar Base (CM0877)

Esculin	0.4 g
Ammonium iron (III) citrate	0.25 g
Mannitol	5.0 g
Glucose	0.25 g
Lithium chloride	7.5 g
Peptone	11.5 g
Phenol red	0.04 g
Starch	0.5 g
Sodium chloride	2.5 g
Agar	6.5 g

Motility Test Medium

Motility test medium	8 g
Nutrient broth	2 g
Sodium chloride	0.5 g
Distilled water	500 mg

D10 media (mg l^{-1}) and MCDB 202

Content	MCDB202	D10
Vitamins		
Biotin	0.00733	Nil
Choline Chloride	13.39	Nil
Vitamin B12	0.1355	Nil
Folinic acid, calsium	0.0006016	Nil
Myo-Inositol	18.02	Nil
Nicotineamide	6.10	Nil
D-Panthothenic Acid	0.2383	Nil
Pyridoxine.HCl	0.0616	Nil
Riboflavin	0.1129	Nil
Thiamine.HCl	0.337	Nil
Inorganic salts		
Ammonium Metavandate	0.000585	Nil
Ammonium Molybdate	0.0012359	Nil
Calcium Chloride.H_2O	22.0	Nil
Cupric Sulphate.$5H_2O$	0.0002479	Nil
Potassium Chloride	186.25	Nil
Potassium Phosphate	68.05	8500
Sodium Chloride	7183.25	Nil

Table* for D10 media (mg l^{-1}) and MCDB 202 *cont.d

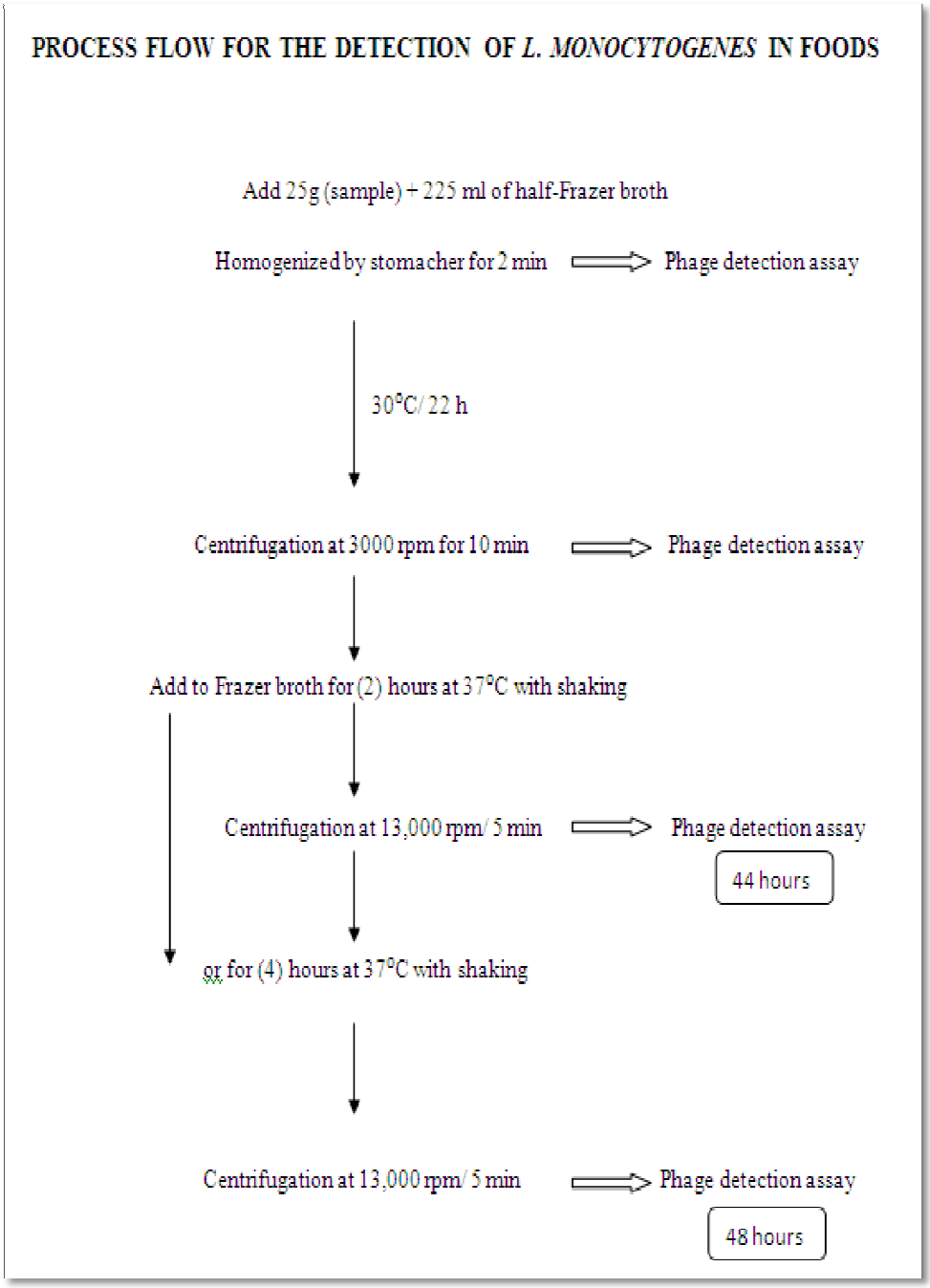
Sodium Metasilicate.$9H_2O$	0.1421	Nil
Sodium Phosphate dibasic	71.05	1
Sodium Selenite	0.00789	Nil
Stannous Chloride dihydrate	0.000001128	Nil
Zinc Sulphate.$7H_2O$	0.02875	Nil
Ammonium Chloride	Nil	500
Ferric Chloride	Nil	48
Amino Acids		
L-Alanine	8.90	Nil
L-Arginine	52.26	200
L-Aspargine	132.10	Nil
L-Aspartic acid	13.31	Nil
L-Cysteine	24.44	100
L-Glutamic acid	14.71	Nil
L-Glutamine	146.00	Nil
Glycine	7.51	Nil
L-Histidine	15.52	200
L-Isoleucine	13.12	200
L-Leucine	39.36	100

Table* for D10 media (mg l^{-1}) and MCDB 202 *cont. d

L-Lycine	29.24	Nil
L-Methionine	4.48	200
L-Phenylalanine	4.96	Nil
L-Proline	5.76	Nil
L-Serine	31.53	Nil
L-Threonine	35.73	Nil
L-Tryptophane	6.13	Nil
L-Tyrosine	9.06	Nil
L-Valine	35.16	200
Other		
Adanine	0.135	Nil
Thymidine	0.07266	Nil
D-Glucose	1440	1000
Linoieic Acid	0.0561	Nil
Lipoic Acid	0.00206	0.0001
Phenol Red, Sodium	1.242	0.0001
Puytrecine.2HCl	0.0001611	Nil
Sodium Pyruvate	55.00	Nil
Riboflavin	Nil	1.0
Thiamine	Nil	1.0

Biotine	Nil	0.01

APPENDIX 2


PROCESS FLOW FOR THE DETECTION OF L. MONOCYTOGENES IN FOODS
Add 25g (sample) + 225 ml of half-Frazer broth
Homogenized by stomacher for 2 min
Phage detection assay
30°C/ 22 h
Centrifugation at 3000 rpm for 10 min
Phage detection assay
Add to Frazer broth for (2) hours at 37°C with shaking
Centrifugation at 13,000 rpm/ 5 min
Phage detection assay
44 hours
or for (4) hours at 37°C with shaking
Centrifugation at 13,000 rpm/ 5 min
Phage detection assay
48 hours

APPENDIX 3

PHAGE DETECTION ASSAY FOR *L. MONOCYTOGENES* AND *LISTERIA* SPP. GROWN IN DIFFERENT MEDIA