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**The Effect of Growth Conditions on the
Surface Properties of *Listeria
monocytogenes***

By

Ogueri Nwaiwu

**A Thesis submitted to
the University of Nottingham
for the degree of Doctor of Philosophy**

**Division of Food Sciences
School of Biosciences**

**UNIVERSITY OF NOTTINGHAM
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July 2011

This thesis is dedicated to Almighty God

**The Effect of Growth Conditions on the Surface
Properties of *Listeria monocytogenes***

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ABSTRACT

Due to the recent persistence of *Listeria monocytogenes* in food factory environments and an increase in outbreaks of Listeriosis, in particular some associated with duck meat products, an investigation was carried out to establish the potential effects of different growth conditions on the hydrophobicity of *L. monocytogenes* cells and to determine the behaviour of the cells in a minimal nutrient environment. It was found that duck meat extracts increased growth rate but did not alter the surface charge of the cells and when grown in minimal D10 and MCDB202 media the cells flocculated and showed more hydrophobicity than when grown in the rich media BHI. The modified surface of the organism behaved like an emulsifier and this led to the discovery for the first time, that there was formation of capsular exopolymeric substances (EPS) on the surface of planktonic cells of *L. monocytogenes*. After confirmation of the capsular EPS by two capsule stains, namely Nigrosin and Giemsa, the EPS was purified and proved to play a role in holding the cells together. It was also found to absorb water rapidly and can retain water for long periods suggesting that the EPS can contribute to the desiccation tolerance of *L. monocytogenes* cells embedded in a biofilm matrix. Chemical characterization of the EPS showed high levels of glycerol and phosphate indicating that the EPS is amphiphatic and may contain mainly glycerolphosphates.

CHAPTER 1

INTRODUCTION

1.0 INTRODUCTION

1.1 *Listeria monocytogenes*

Since first described by Murray *et al.* (1926), this organism has continued to show worldwide prevalence associated with serious diseases in man and a wide variety of animals (Gray and Gillinger, 1966). It is a Gram-positive non-spore forming bacterium (Djordjevic *et al.*, 2002; Fugget *et al.*, 2007) and a facultative intracellular pathogen but exists as a gut commensal and is also found commonly in soil, surface water, sewage, vegetation and food processing plants (Nightingale *et al.*, 2007, Van der Veen and Abee, 2010). Hence it is often found in unprocessed food of animal origin such as fish, milk, meat and poultry and also in contaminated vegetables or salad (Anon^a, 2005).

It is a rod shaped bacteria 0.5 μm in width and 1-1.5 μm in length and with a low G+C content (Liu, 2006). On Brain heart infusion agar the colonies are small round and a light milky white colour when illuminated with reflected light. Even though it has optimum temperature range of 30-37 $^{\circ}\text{C}$, as a psychrotroph it can grow over a wide temperature range between 3 and 45 $^{\circ}\text{C}$. The organism has the ability to multiply rapidly in aerobic or micro-aerophilic environments and will tolerate a wide range of pH (McClure *et al.*, 2008) conditions (between pH 4 and 9).

L. monocytogenes has been identified throughout the environment, in the soil, water and decaying vegetation (Welshimer, 1968). It was found by Watkins and Sleath (1981) that viable counts of *L. monocytogenes* from sludge sewage sprayed on agricultural land remained unchanged for 8 weeks. Its ability to degrade plant material is relevant to its survival since this bacterium is able to utilize nutrients available in the soil (Gray and Killinger, 1966).

1.2 Epidemiology of Listeriosis

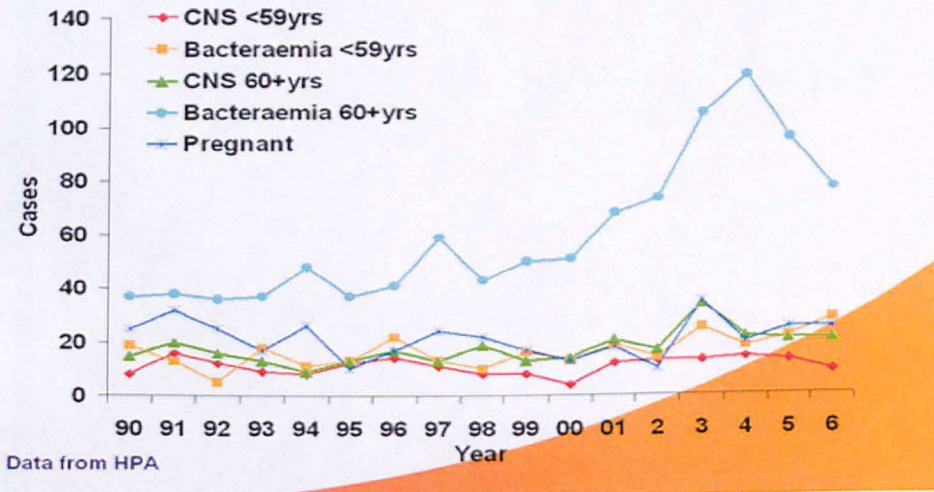
In a survey in 2000 among other prevalent food pathogens that included *Escherichia coli*, *Campylobacter*, *Salmonella* and *Clostridium*, *L. monocytogenes* resulted in the highest rate (100%) of hospital admissions (Adak *et al.*, 2002). This is because diagnosis normally occurs only when severe health problems have set in and the disease is no longer self-limiting.

The incidence of Listeriosis in England and Wales has been on the rise in recent years (Fig. 1; Anon^b, 2008) with the number of non-pregnancy associated cases being greater than pregnancy associated cases of Listeriosis. Also there has been an unexplained increase in the number of persons over 60 years infected with the organism in the last decade. Outside the UK, the bacterium is reported to cause 2500 cases of human illness and 500 deaths in America annually (Cai *et al.*, 2002). The disease is described as serious infection caused by eating food contaminated with *L. monocytogenes* and in 2008, an outbreak of *Listeriosis* in Canada resulted in 22 deaths out of 57 reported (Anon^c, 2009).

The disease Listeriosis affects primarily pregnant women, the new borne and adults with weakened immune system (see Low and Donachie, 1997) since *L. monocytogenes* is an intracellular pathogen, cell-mediated immunity is required to clear infection. In healthy adults, and in animals, the symptoms include fever, muscle, aches and sometimes nausea and diarrhea but are generally self-limiting and the organism is cleared from the body. In immuno-compromised individuals this can progress to encephalitis, meningitis, abortion (in pregnant women) and septicemia. Headache, stiff neck, confusion and loss of balance are symptoms seen when infection spreads to the nervous system.

Figure. 1.1 Epidemiological data for Listeriosis

Changing pattern of listeriosis in England and Wales, numbers of cases reported



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<http://www.hpa.org.uk/webw/>

Human cases of Listeriosis showing that non pregnancy associated cases occurred more than pregnancy associated cases. There has been significant increase in number seen from 2000. Data collected as part of the National Surveillance by the UK Health Protection Agency

Before Listeriosis was recognized as a major food borne pathogen, Gray and Gillinger (1966) observed that in most reported cases of Listeriosis there was no direct contact with animals. However following investigations of large outbreak of Listeriosis, epidemiological and laboratory evidence was used to confirm the suspicion that Listeriosis was a food borne disease (Schlech *et al.*, 1983 and Linan *et al.*, 1988). Schuchat *et al.* (1991) have shown that the epidemiological pattern of Listeriosis can be sporadic, nosocomial or food borne.

It has been suggested that the intestinal tract is the portal entry of *Listeria* infections. Riedel *et al.* (2009) pointed out that during its parasitic lifecycle, *L. monocytogenes* copes with a series of challenges which arise during different stages of infection. These challenges include low nutrient availability, resistance to various physical stress conditions (e.g. temperature, acid, bile; Gahan and Hill, 2005) and the host immune system (Pamer, 2004) and hence is able survive passage through the GI tract and cause both infection and colonisation.

Schuchat *et al.* (1991) studied fecal carriage in a number of populations and it was found that stool cultures from healthy slaughter house workers, hospitalized adult patients with diarrhea and people that made contact with *Listeria* patients had different levels of *Listeria* in their stools. The percentage of carriage was 4%, 1.2%, 1% and 26%, respectively, in the three groups studied.

1.3 Detection and identification of *Listeria*

According to Schmid (2005) the genus *Listeria* contains the two pathogenic species *Listeria monocytogenes* and *Listeria ivanovii* and the four apparently non pathogenic species *Listeria innocua*, *Listeria seeligeri*, *Listeria welshimeri*, and *Listeria grayi*. The pathogenicity of the former two species is due to an approximately 9 kbp virulence gene cluster which is also present in a modified form in *L. seeligeri*. Among the six species in the genus only *L. monocytogenes* is a common pathogen for humans (Zhang *et al.* 2010), but rare cases of infection caused by *L. ivanovii* have been reported.

Gray *et al.* (2004) reported that *L. monocytogenes* strains group into two major divisions, designated lineages I and II. Strains of serotypes 1/2b, 4b, 3b, and 3c consistently group into lineage I, while serotype 1/2a, 1/2c, and 3a strains group into lineage II (Nadon *et al.*, 2001). The *L. monocytogenes* lineage III strains represent a third, distinct taxonomic group which predominantly includes serotype 4a and 4c strains (Wiedman *et al.*, 2002). Majority of sporadic human Listeriosis cases appear to be caused by serotype 4b and 1/2b strains, while most human Listeriosis outbreaks have been caused by serotype 4b strains (Wiedman *et al.*, 1997).

According to Jeffers *et al.* (2001), host specificities have been identified in other bacterial pathogens that affect man and animals but this has not been demonstrated for different *L. monocytogenes* strains which seem to be able to infect man and animals equally well. In contrast *L. ivannovi* shows a lower virulence in man compared to animals; hence infections caused by this organism in humans are rare.

1.4 Molecular typing of *Listeria monocytogenes*

According to Gasanov (2005) after isolation, selective enrichment and plating and completing further tests to confirm identity, specialized typing methods are required to sub-type organisms for epidemiological research. Those used for *Listeria* include serotyping (Unnerstad *et al.*, 1999), phage typing (Capita *et al.*, 2002), multilocus enzyme electrophoresis (Baloga and Harlander, 1991; Norrung, 1992), ribotyping (Bruce *et al.*, 1995) PCR-RFLP (Bille and Rocourt, 1996 and Sauders *et al.*, 2004), RAPD (Mazurier *et al.*, 1992 and Czajka and Batt, 1994), PFGE (Destro *et al.*, 1996; Maule, 1998) and Microarray (Call *et al.*, 2003).

1.4.1 Ribotyping, lineage analysis and molecular serotyping

Ribotyping is restriction fragment length polymorphism analysis of rRNA genes that are used for differentiation between species of strains. Normally genomic DNA is digested and separated by gel electrophoresis. Universal probes that target specific conserved domains of RNA coding sequences are used to detect band patterns (Bouchet *et al.*, 2008).

Wiedmann *et al.* (1996) used ribotyping to show that a diverse population of *L. monocytogenes* strains existed in farm environments of which some may be more likely than others to cause disease. They investigated four out breaks and found that in all but one outbreak, *L. monocytogenes* strains represented by the same ribotype were isolated from both clinical and silage samples. They used ribotyping because it had been found to be a reliable and reproducible typing method for which a large data base exists (Bruce, 1995) and also it can identify clonality within populations allowing specific strains to be tracked.

However, pulsed field gel electrophoresis is a more discriminative method than ribotyping (i.e. allowed more sub-types of strains to be identified). In this method large pieces of DNA are separated by alternating direction electrical which cannot be resolved using conventional gel electrophoresis (Latorre *et al.*, 2009).

Results from various sub typing studies have shown that the 13 serovars of *Listeria* can be divided into three distinct evolutionary lineages (Roberts *et al.*, 2006). Piffaretti *et al.* (1989) first used multilocus enzyme electrophoresis to identify and classify *L. monocytogenes* into two divisions. This was confirmed by results obtained for the partial sequencing of the listeriolysin virulence gene, *hly* (Rasmussen *et al.*, 1991). Further confirmation of the two lineages

was shown by both Brosch *et al.* (1994) who used pulsed field gel electrophoresis and Graves *et al.* (1994) using ribotyping.

The first evidence of a third *L. monocytogenes* phylogenetic lineage was provided by Rasmussen *et al.* (1995) who analyzed partial DNA sequences of the flagellin gene, *flaA*, and the virulence genes *iap* and *hly*. The existence of this third lineage was confirmed by ribotyping and by PCR-RFLP (Restriction Fragment Length Polymorphism) analysis of *L. monocytogenes* virulence genes, comparative genetics and sequencing studies (Wiedmann *et al.*, 1997; Doumith *et al.*, 2004; Ward *et al.*, 2004). The fact that several different approaches led to the identification of the same grouping indicates that these are robust conclusions.

Nadon *et al.* (2001) pointed out that conventional serotyping correlates well with these lineage assignments with lineage I including isolates belonging to serotypes 1/2b, 3b,3c and 4b while serotypes 1/2a, 1/2c and 3a are in lineage II and Serotypes 4a and 4c in lineage III. Lineage III has been further classified into three distinct sub groups termed IIIA, IIIB and IIIC by Roberts *et al.* (2006). They analysed partial *sigB* and *actA* sequences and found that the sub-groups occupy different ecological niches and therefore may be transmitted by different pathways, hence explaining why they are not commonly associated with food borne disease.

Doumith *et al.* (2004) and Zhang and Knabel (2005) have pointed out that *L. monocytogenes* serotypes 1/2a and 4b are responsible for most cases of human Listeriosis worldwide. Since classical serotyping is laborious, they developed a multiplex PCR assay to identify and differentiate the different *Listeria* serotypes. Doumith *et al.* (2005) later validated this assay in several laboratories in order to develop it as an international standard. More recently Nightingale *et al.* (2007)

demonstrated that combining *sigB* allelic typing and multiplex PCR improved the discriminatory power and reliability of *L. monocytogenes* molecular serotyping methods and these are now accepted as reliable replacements of the classical serotyping procedures.

From previous epidemiological studies, it has been found that isolates from human Listeriosis cases are over represented in lineage I, while lineage II strains are prevalent in food isolates and lineage III in isolates from cases of animal clinical Listeriosis (Nightingale, 2007). Jeffers *et al.* (2001) observed that even though lineage III are also able to cause human disease, they are more adapted to infection of mammalian hosts other than humans although, as suggested by Roberts *et al.* (2006), this may just reflect the adaptation of these strains to different environments where they are more likely to be ingested by animals.

1.5 Growth of *L. monocytogenes* on food products

It is an established fact that *L. monocytogenes* grows well on meat and poultry products. This is in part due to the organism's ability to grow in a wide range of physiological and temperature conditions but also due to contamination during processing and subsequent growth of the *Listeria* in products during storage (Mead *et al.*, 2006).

For instance Jay (1996) studied the prevalence of *Listeria spp* in meat and poultry products and found that although the prevalence was variable, fresh meat tended to contain a lower microbial load than processed meat products and this increase in numbers suggests contamination of product is occurring during processing.

Similarly Mahmood *et al.* (2003) examined meat products from different poultry shops and found that the incidence of *L. monocytogenes* was much higher in frozen poultry meat products than in fresh poultry meat samples, while Burnett *et al.* (2005) studied the

growth or survival of *L. monocytogenes* in cold-smoked salmon; sliced, cooked ham; sliced, roasted turkey; shrimp salad; and coleslaw obtained at retail supermarkets stored at 5 °C, 7 °C, or 10 °C (41 °F, 45 °F, or 50 °F, respectively) for up to 14 days. They found a decline in populations of *L. monocytogenes* was observed in shrimp salad and coleslaw, and the rate of decline was influenced by storage temperature. They concluded that the growth of *L. monocytogenes* in potentially hazardous ready-to-eat foods is dependent upon the composition of individual food products, as well as storage temperature.

Rorvik *et al.* (2003) have observed that growth of *L. monocytogenes* is very prolific in poultry when 385 poultry products samples were screened for *L. monocytogenes*. They characterized the recovered isolates and 19 patient isolates by multilocus enzyme electrophoresis and restriction enzyme analysis. The poultry isolates showed great genetic diversity, but no identical sub clones were identified from poultry sources and patients. When one slaughterhouse was examined in detail during a 16-month period, they found that contamination rates increased along the processing line, and one sub clone was found during the whole period. They advised that preventive measures to avoid contamination of poultry products by *L. monocytogenes* must be taken in the processing plant.

Barbalho *et al.* (2005) investigated the occurrence of *Listeria* contamination in an industrial poultry processing plant by sampling carcasses at varying stages of processing. *Listeria* was isolated at all sampling sites and the species most often isolated was *L. innocua*, which accounted for 28 of the 31 (90.3%) isolates. The frequency of *Listeria* in the chicken carcasses was similar at bleeding, defeathering and end of evisceration stages (33.3%), and it reduced during scalding (16.7%), and rose immediately after initial evisceration stage (50%) to peak after packaging (76.2%).

Also Miettinen *et al.* (2001) sampled the environment and products from two broiler abattoirs and processing plants and raw broiler pieces at the retail level for *L. monocytogenes* and found that the *L. monocytogenes* contamination rate varied from 1 to 19% between the two plants studied. Furthermore, 62% (38 of 61) of the raw broiler pieces, bought from retail stores, were positive for *L. monocytogenes*. Altogether three serotypes (1/2a, 1/2c, and 4b) and 14 different PFGE types were obtained.

In addition to the association of *Listeria* with particular food products, it has now been shown that there is persistence of serotypes of *L. monocytogenes* associated with human disease in food retail environment (Sauders *et al.*, 2004). This persistence has been associated with biofilms (see section 1.7).

1.6 Growth and survival of *Listeria monocytogenes* in minimal nutrient environment

Since *L. monocytogenes* has the ability to survive in an environment with minimal nutrient availability (Abram *et al.*, 2008), there is the need to understand how they behave in such environment.

Friedman and Roessler (1961) looked at the growth requirements of three strains of *L. monocytogenes* and found that they had different vitamins and amino acid requirements. Welshimer (1963) showed that *L. monocytogenes* failed to grow in the absence of riboflavin, biotin, thiamine and theistic acid and using this information several minimal media have been developed. For instance Trivet and Meyer (1971) developed a synthetic growth medium D10 and using this found that *L. monocytogenes* uses a split non-cyclic citrate pathway which has an oxidative and reductive portion. It is believed by many that this D10 media is still the best defined media developed for the minimal cultivation of *Listeria*, however the large number of components required does make this a difficult media to prepare.

To simplify the preparation of minimal media, Premaratne *et al.* (1991) modified the media of Welshimer (1963) who observed that *L. monocytogenes* failed to grow in the absence of riboflavin, biotin, thiamine and theistic acid. Phan-Than and Gormon (1997) pointed out that complex media like Brain Heart Infusion (BHI) is not suitable for understanding how *Listeria* behaves in a minimal nutrient environment since its composition is not consistent. They modified the minimal media described by Premaratne *et al.* (1991) by incorporating additional essential vitamins and growth factors to obtain an optimal growth of *L. monocytogenes* in a minimal chemical defined medium.

Listeria is exposed to multitude of adverse environmental conditions and can be stressed by sanitizers, preservatives, heat, cold or freezing temperatures, drying and osmotic changes (Vogel *et al.*, 2010). Exposure to any such stresses can contribute to the enhanced survival and persistence of *Listeria*.

Alonso-Hernando *et al.* (2009) reported that *L. monocytogenes* can adapt to sub-lethal concentrations of chemicals when incubated in broths of increasing sub-lethal concentration. They repeatedly exposed *L. monocytogenes* to increasing sub-inhibitory concentrations of trisodium phosphate, acidified sodium chlorite, citric acid, chlorine dioxide or peroxyacetic acid (decontaminants used in poultry). They compared the antibiotic resistance patterns of the strains before and after exposure to decontaminants and found increases in resistance to antibiotics by *L. monocytogenes*.

Murphy *et al.* (2003) showed that there were significant differences in the heat resistance of *L. monocytogenes* found in turkey, duck and chicken products. Cells in turkey had the highest D value (i.e. longest time to reduce the population by one log₁₀ value) followed by duck muscle and duck skin while chicken had the lowest D value. They also found significant differences for the heat resistance of *L. innocua* and *L. monocytogenes* among turkey, duck, and chicken products

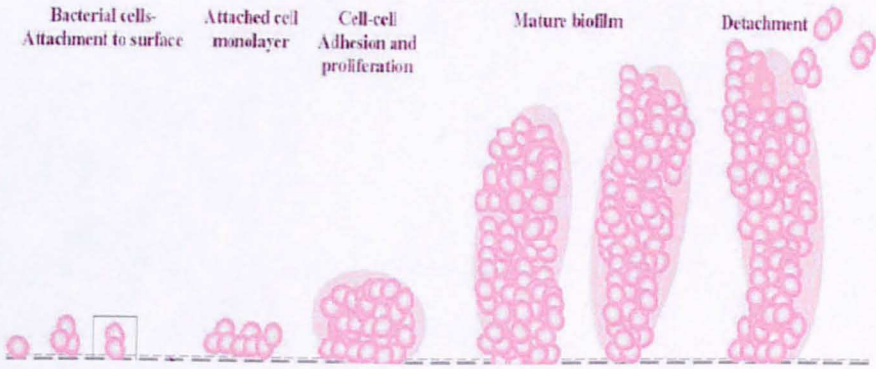
which and concluded that the kinetic values of a certain pathogen in a specific product should be used for determining process lethality in fully cooked and vacuum-packaged poultry products during post-cook heat treatments

1.7 Formation of Biofilms.

Biofilms are generally referred to as an accumulated mass of bacteria and their extracellular matrix on a solid surface (O' Toole *et al.*, 2000). Bacteria cell attachment to a surface has been shown to initiate biofilm formation (Palmer *et al.*, 1997). Hall-Stoodley *et al.* (2004) reported that biofilms have probably existed since prehistoric times (3.4 billion years ago) and evolved as part of the ability of microbes to cope with the harsh environmental extremes of earth present at that time. Biofilms normally form when cells first attach to a surface and then start to proliferate, before finally the biofilm reaches maturity as shown in Figure 1.2 (Katsikogianni and Missirlis, 2004).

Prakash *et al.* (2003) pointed out that the structured biofilm-forming microorganisms elicit special mechanisms for initial attachment to take place. Once attached the cells enclose them in a self produced polymeric matrix that allows survival in a hostile environment. Donlan and Costerton (2002) reported that the process of bacteria adhesion includes a reversible physical phase (Phase1) followed by an irreversible molecular and cellular phase (Phase2). In phase one bacterial adhesion to surfaces consists of an initial attraction of the cells to the surfaces followed by adsorption and attachment while in phase 2 molecular-specific reactions between bacteria surface structures and substratum surface structures become predominant.

Figure 1.2 Stages of biofilm formation



Planktonic cells attach to a surface to initiate biofilm development. This is followed by cell-to-cell adhesion as growth is initiated before the cells proliferate in a three dimensional matrix to form a mature biofilm. Finally cells can detach from the biofilm to re-seed the environment with planktonic cells. Taken from Katsikogianni and Missirlis (2004).

There is also a firmer adhesion of bacteria to a surface by selective bridging function of bacterial surface polymeric structures like capsules, pilli and slime and also bacterial cell hydrophobicity (Vacheethasanee *et al.*, 1998). Hence properties of the cell surface that can influence the attachment of bacteria include surface appendages (Pratt and Kolter 1998 and Beloin *et al.* 2004) and surface polysaccharide (Schembri *et al.* 2003).

Hood and Zottola (1995) pointed out that hydrophobic interactions are responsible for much of adherence of cells to surfaces. Husmark and Ronner (1992) showed that bacterial spores generally attach to surfaces at a high rate than vegetative cells due to the higher hydrophobicity of spores while Zita and Hammerston (1997) suggested that cell surface hydrophobicity plays an important role in the attachment of *Escherichia coli* to surfaces.

Katsikogianni and Missirilidis (2004) explained that other factors that affect bacteria adhesion include the surface characteristics of the material and the presence of organic material bound to that surface, such as serum proteins. Material surface characteristics influencing bacterial adherence include chemical composition (Cordero *et al.* 1996) surface charge (Gottenbos *et al.* 2002) and surface roughness (Scheuerman *et al.* 1998). Other environmental factors that affect the rate of biofilm formation include temperature, time of exposure to planktonic cells, concentration of bacteria in the environment and associated flow conditions.

According to Hu and Ehrlich (2008), it has become clear that over the past decade and half that bacteria exhibit many of the hallmarks of a multicellular organism when growing as biofilms and communicating among each other using quorum sensing systems (Fuqua *et al.*, 1994). Erlich *et al.* (2002) pointed out those bacteria that are exquisitely susceptible to low concentrations of antibiotics when growing planktonically can become highly resistant to the most potent, latest generation broad spectrum antibiotics when living as a community within biofilms and therefore the cells embedded in this matrix are protected from the external conditions.

1.8 Attachment of *Listeria* to surfaces

According to Hood and Zottola (1995) when biofilm forms on food processing surfaces, it results in persistent populations. Persistence of *Listeria* in a food processing environment can range from 8 months to 10 years (Lunden *et al.*, 2002). Furthermore Lunden *et al.* (2000) found that such persistent strains have the added ability of enhanced adhesion within shorter times to surfaces compared to non-persistent strains.

Pan *et al.* (2006) simulated a food processing environment and suggested that the resistance of *L. monocytogenes* biofilms to sanitizing agents may be due to attributes of the biofilm matrix rather than being an intrinsic attribute of the cell in the biofilm.

Despite the fact that *Listeria* is associated with biofilm formation in food environments, it is not classically a good biofilm former. Harvey *et al.* (2007) assessed biofilm formation of 138 *L. monocytogenes* strains and observed that only 2 can be classified as strong biofilm formers. However Treese *et al.* (2006) noted that preservation of food product at pH 5 delayed bacterial adhesion and biofilm production during food processing, again indicating that the specific growth conditions used in laboratory studies of *Listeria* may affect the results gained and hence may not reflect behavior of this organism in the food environment.

Unlike many of the closely related *Bacillus* species (see Sallen 1996), the formation of capsular material by *Listeria* that would form the major component of the biofilm matrix has not been reported. Studies have focused on the *Listeria* biofilm matrix as a whole but not the specific extracellular polymeric substance (EPS; see section 1.9) housing the biofilm. However, attempts have been made to identify other compounds necessary for the formation of EPS/biofilm matrix.

Rieu *et al.* (2008) characterized the structural dynamics of *L. monocytogenes* EGD-e sessile growth in two nutritional environments (with or without a nutrient flow) and found that in the absence of nutrient flow (static conditions), unstructured biofilms composed of a few layers of cells that covered the substratum were observed. In contrast, when grown under dynamic conditions, *L. monocytogenes* EGD-e biofilms were highly organized. They observed ball-shaped microcolonies surrounded by a network of knitted chains.

Abachin *et al.* (2002) reported that Formation of D-alanyl-lipoteichoic acid is required for adhesion and virulence of *L. monocytogenes*. They characterized the *dlt* operon of *L. monocytogenes* that catalyses the incorporation of D-alanine residues into the cell wall-associated

lipoteichoic acids (LTAs). The characterization was carried out by constructing a D-Ala-deficient LTA mutant by inactivating the first gene (*dltA*) of this operon. The virulence of the DltA⁻ mutant was severely impaired in a mouse infection model and, *in vitro*, the adherence of the mutant to various cell lines was strongly restricted. They suggested that the decreased adherence of the DltA⁻ mutant to non-phagocytic and phagocytic cells might be as a result of the increased electronegativity of its charge surface.

Boruki *et al.* (2003) screened eighty *L. monocytogenes* isolates for biofilm formation to determine if there is a robust relationship between biofilm formation, phylogenic division, and persistence in the environment. They detected significant differences between phylogenetic divisions and increased biofilm formation was observed in Division II strains (serotypes 1/2a and 1/2c), which are not normally associated with food-borne outbreaks. Differences in biofilm formation were also detected between persistent and non-persistent strains isolated from bulk milk samples, with persistent strains showing increased biofilm formation relative to non-persistent strains. They found no significant differences among serotypes.

Harmsen *et al.* (2010) showed that *L. monocytogenes* extracellular DNA (eDNA) may be the only central component of the biofilm matrix and that it is necessary for both initial attachment and early biofilm formation when they tested 41 *L. monocytogenes* strains. DNase I treatment resulted in dispersal of biofilms, not only in microtiter tray assays but also in flow cell biofilm assays but no explanation of the origin of this DNA was given.

It has been suggested by Habimana *et al.* (2009) that little is known about the biofilm properties that influence the initial attachment of *L. monocytogenes* to the biofilm interface, but rather they found that presence of EPS from other bacteria promotes attachment.

1.9 Major problems of bacteria attachment and biofilm formation in food industries

In their review Van Houdt and Michiels (2010) reported that the ability of many bacteria to adhere to surfaces and to form biofilms has major implications in a variety of industries including the food industry, where biofilms create a persistent source of contamination. It has been reported that the attachment of bacteria and subsequent development of biofilms is a potential source of contamination of finished products that may shorten shelf life or result in transmission of diseases (Sharma and Anard, 2002).

Other problems include spoilage, increase in corrosion process, reduced flow and blockage of pipes, encouraging the growth of other pathogenic organisms and contamination of food processing surfaces. Furthermore, they cause impedance of heat transfer processes and cause increase in fluid frictional resistance. The formation of a biofilm is determined not only by the nature of the attachment surface, but also by the characteristics of the bacterial cell and by environmental factors.

Kim and Frank (1994) found that *L. monocytogenes* cells grown in D10 showed 50 fold higher attachment on stainless steel than those grown in Tryptone Soy Broth (TSB), again demonstrating the need to grow *Listeria* cells in an environmentally-relevant media to ensure that results gained are relevant to the real world situation.

For *L. monocytogenes*, flagellae have been shown to be involved in the initial attachment to surfaces. Todhanakasem and Young (2008) exploited two different approaches; the use of flow cells and the static-microtiter-plate assay, to gain insight into *L. monocytogenes* biofilm development. They found that the use of the static-microtiter-plate assay revealed that flagella motility contributes to biofilm formation while the use of flow cells combined with microscopy revealed that *L. monocytogenes* biofilm formation, displays stages of biofilm

development. Also, Vatanyoopaisarn *et al.* (2000) showed that at 22 °C a flagellin mutant of *L. monocytogenes* was found to attach to stainless steel at levels 10-fold lower than wild-type cells, even under conditions preventing active motility. However, they found that at 37 °C, when flagella are not produced, attachment of both strains was identical which confirmed that flagella facilitate the early stage of attachment.

For environmental factors, especially in food-processing environments, it was reported that bacterial attachment is additionally affected by food matrix constituents. Residues from ready-to-eat meat products, such as small amounts of meat extract, frankfurters or pork fat, initially reduced the biofilm formation of *L. monocytogenes*, but with time this residual material supported increased biofilm cell numbers and prolonged survival of the bacterium on a variety of materials including stainless steel, conveyor belt rubber, and wall and floor materials (Somers and Wong, 2004).

Similarly Allan *et al.* (2004a; 2004b) showed that survival rates of *L. monocytogenes* on several surfaces, including stainless steel, acetal resin, mortar and fibreglass reinforced plastic, were higher in the presence of biological soil and skim milk and milk proteins such as casein and lactalbumin were found to significantly reduce the attachment of vegetative cells of *L. monocytogenes* to stainless steel (Helke *et al.*, 1993; Barnes *et al.*, 1999; Parkar *et al.*, 2001).

Another factor affecting the formation of biofilms is the presence of other bacterial cell types which can either promote or interfere with the development of *Listeria* biofilms. Hence the presence of *Staphylococcus xylosus* was found to affect the numbers of *L. monocytogenes* found in biofilms on stainless steel (Norwood and Gilmour, 2001).

Similarly, bacteriocin-producing *L. lactis*, as well as several endogenous bacterial strains isolated from food-processing plants,

influenced the establishment of *L. monocytogenes* on stainless steel, suggesting that the resident microflora can have a strong suppressing effect on *L. monocytogenes* establishment in biofilms in a food-processing environment (Leriche *et al.*, 1999; Carpentier and Chassaing, 2004).

1.10 Exopolymeric substance (EPS)

Geesey (1982) described EPS as extracellular polymeric substance of biological origin that participate in microbial aggregates. The abbreviation is commonly used for extracellular polysaccharides and exopolymers. In early biofilm studies, exopolysaccharides was the predominant substance of EPS in bacteria (Costerton *et al.*, 1987) and that is why EPS was used for exopolysaccharides.

However Wingender *et al.* (1999) pointed out that EPS is best used for extracellular polymeric substances as a more general and comprehensive term which includes the different classes of organic macromolecules that have been shown to occur in the intercellular spaces of microbial aggregates. This may include polysaccharides, proteins, DNA, nucleic acids, phospholipids and other polymeric compounds.

1.10.1 Production of EPS by Bacteria

Sutherland (1984) and Marshall (1985) pointed out that EPS are important mediators in the adhesion of bacteria and other microorganisms to surfaces. Stewart *et al.* (1995) found that cell and EPS distributions did not always overlap. Therefore, large areas of biofilm may be devoid of any cellular material but consist mainly of EPS.

The location of EPS is outside the cell surface and the extracellular location may be the result of many processes which include active secretion, adsorption from the environment, cell lysis, shedding of cell surface material and active secretion (Wingender *et al.*, 1999; Flemming, 2007). If produced intracellularly the EPS is normally

translocated across the cell membrane into the surrounding medium by various pathways.

The pathway for excreting proteins across membranes have been described by Hueck (1998) while Roberts (1996) described that of polysaccharides. Furthermore, Cadieux *et al.* (1983) found that lipopolysaccharides from the outer membrane of bacteria can also be released spontaneously as EPS.

1.10.2 Importance and function of EPS

Bacteria EPS such as xanthan, gellan cellulose have found application in the food industry. Also, Poly-gamma glutamate (PGA) of *Bacillus* is widely used in the food and chemical industries. Costerton *et al.* (1987) and Roberts (1996) highlighted that in infection processes, EPS in the form of capsular and slime polysaccharides may represent virulence factors, mediating adhesion to tissue surfaces and protecting the invading bacteria from host defense mechanisms. This is achieved by allowing the bacteria to live and grow in a microcolony or biofilm within the host (Costerton *et al.*, 1999). In addition to serving as the structural elements of biofilms, other functions of EPS proposed by Le Chevallier *et al.* (1988) and Foley and Gilbert (1996) include adhesion to surfaces, providing a protective barrier, retention of water and cell-to- mediating aggregation of flocs and sorption of exogenous organic compounds and inorganic ions.

1.7 AIMS AND OBJECTIVES

This study was initiated as a result of recent observations concerning the persistence of *L. monocytogenes* in food factory environments, specifically a particular problem reported of *Listeria* growth and persistence in a cooked duck product production line (Li, 2004). Therefore, the initial aims and objectives of this study were to:

1. Ascertain the behavior of *L. monocytogenes* in a minimal nutrient environment and how its attachment to surfaces is influenced by duck meat extracts.
2. Investigate factors that determine the endemic nature of food factory isolates.
3. Establish the potential effect of different growth conditions on the hydrophobicity of *L. monocytogenes* cells.

However discoveries made during this investigation led to a new direction focusing on:

4. An investigation of the formation of capsular EPS in *L. monocytogenes*.
5. Identification and characterization of the extracellular polymeric substances found on the surface of *L. monocytogenes* cells.

CHAPTER 2:
MATERIALS AND METHODS

2.1 Media preparation

2.1.1 Brain Heart Infusion agar medium and broth.

The media was prepared by suspending 18.5 g of Brain Heart Infusion Broth (Oxoid) in 500 ml of reverse osmosis water for the broth. The agar medium was prepared by adding 1% bacteriological agar (w/w) before suspending in reverse osmosis water. It was boiled gently to dissolve and sterilized by autoclaving at 121 °C and then cooled to 50 °C before pouring into containers or plates.

2.1.2 *Listeria* selective agar (Oxford formulation)

This was prepared by suspending 27.5 g of the *Listeria* Selective agar base (Oxoid CM856) in 500 ml of reverse osmosis water. It was boiled gently to dissolve and sterilized by autoclaving at 121 °C and then cooled to 50 °C. One vial of *Listeria* selective agar Oxford formulation supplement, (Oxoid SR140) was reconstituted with 5 ml of ethanol and sterile distilled water (1:1) before adding to the agar base and then mixed well before pouring into plates.

2.1.3 Preparation of D10 and Chick Fibroblast Basal Media (MCDB 202)

The D10 defined medium was prepared as described by Trivett and Meyer (1971). To prevent formation of precipitates and to provide for the sterilization of the vitamins by filtration, the medium was assembled as follows. (i) K_2HPO_4 (Fisher Scientific), $NaH_2PO_4 \cdot H_2O$ (BDH) and NH_4Cl (Sigma) were dissolved in 500 ml of reverse osmosis water; (ii) $NaOH$ (Fisher Scientific) and nitrilotriacetic acid (Acros Organics) were dissolved in 40 ml of reverse osmosis water; (iii) $FeCl_3 \cdot 6H_2O$ was dissolved in 40 ml of reverse osmosis water (Fresh solution); (iv) the $FeCl_3 \cdot 6H_2O$ 0.048g (Sigma) solution was mixed with the sodium nitrilotriacetic solution; $MgSO_4 \cdot H_2O$ (Fisher Scientific) was dissolved in 30 ml of reverse osmosis water. (v) the solutions from steps iv and v, the amino acids (Acros Organics) and 390 ml of reverse osmosis water were added to the solution from the first step to give a volume of 1000 ml which was autoclaved at 15 psi for 15 min. Ten ml

of vitamin solution (100x) was added when cool. The pH of the medium was between 7.2 and 7.5.

The vitamin solution (100x) was prepared as follows. A 5-mg amount of α -lipoic acid (Fluka Biochemika) was dissolved in 200 ml of 70 % ethanol. Biotin (Fisher Scientific), 5 mg; 50 mg of thiamine(Sigma) and 50 mg of riboflavin (Sigma) in 125 ml of 95 % ethanol were combined with 2 ml of the α -lipoic acid/ethanol solution. The volume was brought to 500 ml and the filtered through a 0.45 μ l membrane filter (Millipore).

The D10 media was supplemented with 10 ml of a 20% glucose solution sterilized by autoclaving. Glucose replaces lost fluid during autoclaving. The vitamins (Welshimer, 1963), amino acids and ammonium (Friedman and Roessler, 1961). Iron used (Sword, 1966) have been shown to be required for growth by *Listeria*.

The MCDB 202 medium was prepared as described by Chavant *et al.* (2002) by dissolving 9.877 g of MCDB202 media (US Biological) in 900 ml distilled water and stirred gently until completely solubilized. Water was added to bring the solution to 1 liter after which it was supplemented with 1% yeast nitrogen base without amino acids (Difco) and 3.6 g of glucose (Fisher Scientific) per liter and without pH adjustment. The media was then was filter sterilized with 0.2 micron membrane filter.

Contents of both media are listed in table 2.1.

Table 2.1 Contents of commercially prepared MCDB 202 and laboratory prepared D10 media (mg/l).

| Content | MCDB202 | D10 |
|---------------------------------------|----------------|------------|
| <i>Vitamins</i> | | |
| Biotin | 0.00733 | Nil |
| Choline Chloride | 13.39 | " |
| Vitamin B12 | 0.1355 | " |
| Folic acid, calcium | 0.0006016 | " |
| Myo-Inositol | 18.02 | " |
| Nicotineamide | 6.10 | " |
| D-Panthenic Acid | 0.2383 | " |
| Pyridoxine.HCL | 0.0616 | " |
| Riboflavin | 0.1129 | " |
| Thiamine.HCL | 0.337 | " |
| <i>Inorganic salts</i> | | |
| Ammonium Metavandate | 0.000585 | Nil |
| Ammonium Molybdate | 0.0012359 | " |
| Calcium Chloride.2H ₂ O | 22.0 | " |
| Cupric Sulphate.5H ₂ O | 0.0002479 | " |
| Ferric Nitrate.9H ₂ O | 1.39 | " |
| Magnesium Sulphate | 180.57 | 410 |
| Magnesium Sulphate.5H ₂ O | 0.0001205 | Nil |
| Nickel Chloride Hexahydrate | 0.0000011885 | " |
| Potassium Chloride | 186.25 | " |
| Potassium Phosphate | 68.05 | 8500 |
| Sodium Chloride | 7183.25 | Nil |
| Sodium Metasilicate.9H ₂ O | 0.1421 | " |
| Sodium Phosphate dibasic | 71.05 | 1 |
| Sodium Selenite | 0.00789 | Nil |
| Stannous Chloride Dihydrate | 0.000001128 | " |
| Zinc Sulphate.7H ₂ O | 0.02875 | " |
| Ammonium Chloride | Nil | 500 |
| Ferric Chloride | " | 48 |
| Sodium Hydroxide | " | 240 |
| Nitriloacetic acid | " | 480 |
| <i>Amino Acids</i> | | |
| L-Alanine | 8.90 | Nil |
| L-Arginine | 52.26 | 200 |
| L-Asparagine | 132.10 | Nil |
| L-Aspartic acid | 13.31 | Nil |
| L-cysteine | 24.44 | 100 |
| L-Glutamic acid | 14.71 | Nil |
| L-Glutamine | 146.00 | " |
| Glycine | 7.51 | " |
| L-Histidine | 15.52 | 200 |
| L-Isoleucine | 13.12 | 200 |

Table 2.1 cntd

| Content | MCDB202 | D10 |
|--------------------|----------------|------------|
| L-Leucine | 39.36 | 100 |
| L-Lysine | 29.24 | Nil |
| L-Methionine | 4.48 | 200 |
| L-Phenylalanine | 4.96 | Nil |
| L-Proline | 5.76 | " |
| L-Serine | 31.53 | " |
| L-Threonine | 35.73 | " |
| L-Tryptophan | 6.13 | " |
| L-Tyrosine | 9.06 | " |
| L-Valine | 35.16 | 200 |
| Other | | |
| Adenine | 0.135 | Nil |
| Thymidine | 0.07266 | " |
| D-Glucose | 1440 | 1000 |
| Linoleic Acid | 0.0561 | Nil |
| Lipoic Acid | 0.00206 | 0.0001 |
| Phenol Red, Sodium | 1.242 | Nil |
| Puytreine.2HCl | 0.0001611 | " |
| Sodium Pyruvate | 55.00 | " |
| Riboflavin | Nil | 1.0 |
| Thiamine | " | 1 |
| Biotin | " | 0.01 |

2.1.4 Preparation of Duck Juice extract

Duck meat was purchased from Sainsbury. The fatty skin was removed and then the meat was cut into small bits. After boiling for 20 min, 100 g were stomached with 300 ml of sterile distilled water. The mixture was vacuum filtered with Whatman No.4 filter paper and autoclaved at 121 °C for 15 min. The juice was stored in aliquots of 200 ml until use.

2.2 Resuscitation of *Listeria monocytogenes* Strains under study

Strains for this study were originally obtained from Agriculture and Food Science centre, Belfast, Northern Ireland, Gunstone Bakery in Yorkshire and Institute Pasteur, Paris and stored at -80°C. Beads

containing the strains were removed from the -80°C freezer and streaked on to rich BHI media. Colonies that emerged after 24 h incubation at 37 °C were observed. To ensure that there was no contamination during recovery of the organisms, the organisms were Gram stained and grown on *Listeria* selective media. The Gram staining was performed by standard methods using a smear from cells grown on agar. Microscopy was used to visualize results.

2.3 Culture Storage

For long term storage, resuscitated strains were made into 3 copies and stored in 20% glycerol. This was carried out by placing 200 µl of glycerol (Fisher Scientific) into a cryopreservation tube followed by autoclaving at 121 °C for 15 min. After autoclaving 800 µl of overnight culture broth of resuscitated strains was placed into the cryopreservation tube and vortexed for 5 s. Two duplicates were stored in -20 °C while the third copy was stored in -85 °C. For short term storage bacteria cultures were kept on BHI agar plates at 4 °C. This was maintained by sub-culturing on BHI agar plates every 4 weeks.

2.4 *Listeria* Strains used for this study

The *Listeria* strains used are shown in table 2.2

Table 2.2 *Listeria* Strains used

| Strain | Serotype | Source |
|------------------------------|-----------------|------------------|
| Environmental Strains | | |
| Lm000101-0305 | unknown | Pot wash |
| Lm00054-0305 | unknown | vegetable |
| Lm00048-0305 | unknown | Roasted chicken |
| Lm000622-0305 | unknown | Bakery |
| Lm000630-0305 | unknown | Bakery |
| Lm000636-0305 | unknown | Bakery |
| Lm00006-0305 | unknown | Scottish Salmon |
| Lm0006-0305 | unknown | Scottish Salmon |
| Lm00064-0305 | unknown | Fresh Chicken |
| Lm4 | 1/2b | Milk |
| Lm27 | 1/2c | Food environment |
| Reference strains | | |
| Lm23074 | 4b | Clinical |
| Lm 10403S | 1/2a | v |
| LMEGD-e | 1/2a | v |
| LmLO28 | 1/2c | v |
| <i>Listeria innocua</i> | unknown | v |

2.5 Solutions and primers used

The solutions and primers used are shown in Table 2.3 while the primers are shown in table 2.4.

Table 2.3 Table showing solutions prepared and used for the study.

| Solution | Description |
|------------------------------------|--|
| DNA extraction | |
| 1. Lysis buffer - | 25 mM Tris 8.0, 10 mM EDTA, 50 mM sucrose (Fisher Scientific) |
| 2. GES buffer | 5 M Guanidium thiocyanate (Fisher Scientific), 0.5% sarkosyl (Sigma), 0.1 M EDTA |
| 3. PuM buffer | 22.2 g $K_2HPO_4 \cdot 3H_2O$, 7.26g $K_2H_2PO_4$, 1.8g Urea, 0.2g $MgSO_4 \cdot 7H_2O$ in 1 L |
| 4. NaCl buffer | 150 mM NaCl (Fisher Scientific). |
| 5. PBS buffer | Dissolved 1% (w/v) potassium phosphate buffer tablet in distilled water. |
| 6. TE Buffer | 10 mM Tris, 1 mM EDTA pH 8.0 |
| SDS polyacrilamide analysis | |
| 7. Separating gel buffer A; | 0.75 M Tris-HCl, 0.2% SDS; pH 8.8 |
| 8. Separating gel buffer B; | 0.25 Tris-HCl, 0.2% SDS; pH 6.8 |
| 9. Running buffer 10x | 25 mM Tris, 0.19% SDS, 0.192 M Glycine (pH 8.3-8.6 without adjustment) |

Table 2.3 ctd

| Solution | Description |
|--------------------------|---|
| 10. Acrylamide: | Bisacrylamide solution (37.5:1.3%), Acrylamide solution (30%) |
| 11. Stain Solution 1 | 0.25 % Coomassie Blue, 50% methanol, 10% acetic acid |
| 12. Stain solution 2 | 0.5% methylene blue in 3% acetic acid |
| 13. Destain solution | Methanol 20%, Acetic acid 10%. |
| 14. Loading dye | 0.5M Tris-HCl, 20% SDS, glycerol, 10% β - mercaptoethanol, Reverse osmosis water and small spatula of Bromophenol blue. |
| 15. Ammonium persulphate | 10%; 10 g/ml |
| 16. Fixing solution | Ethanol, 75 ml; Glacial acetic acid, 25 ml; water to 250 ml |
| 17. Sensitizing solution | Ethanol, 75 ml; Sodium thiosulphate (5% w/v), Sodium acetate, 17 g; Water to 250 ml; Before use, add 1.25 ml glutardialdehyde (25% w/v) |

Table 2.3 ctd

| Solution | Description |
|-------------------------|---|
| <hr/> | |
| Silver Stain | |
| 18. Silver solution | Silver nitrate solution (2.5% w/v); 25 ml water to 250. Before use, add 0.1 ml formaldehyde (37%) |
| 19. Developing solution | Sodium carbonate (6.25 g); water to 250 ml; Before use add 0.2 ml formaldehyde (37%). |
| 20. Stop Solution | EDTA- NO ₂ .H ₂ O, water to 250 ml |
| 21. Preserving solution | Ethanol, 75 ml; Glycerol (87%) 11.5 ml; water to 250 ml |
| <hr/> | |

TABLE 2.4 Primers used for the study

| Gene | Forward and reverse primers | PCR product (bp) | Putative function | Reference |
|-------------------|--|-------------------------|------------------------------------|------------------------------|
| 1. <i>Imo0737</i> | AGGGCTTCAAGGACTTACCC ACGATTTCTGCTTGCCATTC | 691 | Unknown | Doumith <i>et al.</i> (2004) |
| 2. <i>Imo1118</i> | AGGGGTCTTAAATCCTGGAA CGGCTTGTTCCGGCATACTTA | 906 | Unknown | '' |
| 3. ORF2110 | AGTGGACAATTGATTGGTGAA CATCCATCCCTTACTTTGGA | 597 | Unknown | '' |
| 4. ORF2819 | AGCAAAATGCCAAAACCTCGT CATCACTAAAGCCTCCCATTC | 471 | Putative transcriptional regulator | '' |
| 5. <i>prs</i> | GCTGAAGAGATTGCGAAAGAAG CAAAGAAACCTTGGATTTGCGG | 370 | Putative phosphoribosyl synthetase | '' |

2.6 Growth of *Listeria monocytogenes* in broth of D10, BHI, MCDB202 and media containing D10 with duck juice (DJ+D10) supplements.

To generate starter cells, a loop full of cells were transferred into 10 ml of BHI broth and grown at 37 °C overnight with shaking after which 5 ml were pelleted at 3000 x g. The pellets were washed twice with PBS using the same centrifugation conditions. The washed cells were then inoculated into 100 ml of the four different media mentioned above in a 250 ml conical flask and grown at 37 °C with shaking at 150 rpm. Growth was measured by taking OD_{600nm} readings over time with Cecil 20 spectrophotometer.

The generation time and growth rate of the parent and transformed cells were calculated using the equations (Reed *et al*, 2006) shown below

The Specific growth rate (μ)

$$\mu = \frac{2.303(\log_{10}N_x - \log_{10}N_0)}{t_x - t_0}$$

2.7 Flocculation of cells grown in Different media

In order to determine how cells flocculate in different media, cells were prepared as in section 2.6 before transferring into 10 ml of the different broth (BHI, D10, D10+DJ, MCDB 202) in a test tube and were grown overnight with shaking at 37 °C after which the tubes were left to stand on the bench for one minute and the distribution of the cells were photographed. Further observations were made after 72 h incubation at 37 °C.

2.8 Determination of cell surface charge of broth culture used by measuring the Zeta potential.

Overnight cultures produced as shown in section 2.7 were used. Zeta potential was determined by placing a 5µl of the culture in the measuring port of the zeta potential instrument (Berkman Coulter Ver 1.34). Values shown on the display unit were recorded. Samples were prepared in triplicates.

2.9 Extraction of Genomic DNA

This was carried out as described by Pitcher *et al* (1989). To each of the sterile test tubes, 5 ml of BHI broth was added and inoculated with a colony of *Listeria monocytogenes*. The test tubes were placed in a test tube shaker (New Brunswick Scientific) and incubated at 37 °C overnight. In a sterile eppendorf, 1.5 ml of the overnight broth was added and pelleted with a microfuge (Biofuge 13; Heraeus Sepatech) at 12000 x g for 1 min. The process was repeated to concentrate the cells two fold; following which 1 ml ice cold lysis buffer (25 mM Tris-HCl 8.0, 10 mM EDTA, 50 mM sucrose) was used to wash the cells.

The cells were treated with 50 mgml⁻¹ lysozyme and incubated for 1 h after which 0.5ml lysis solution (5M Guanidium thiocyanate, 0.1 M EDTA, 0.5 % Sakosyl) was added, mixed well and left at room temperature for 5minutes. The lysate was cooled on ice for and 0.25 ml ammonium acetate was added, vortexed and incubated on ice for 10 min. Chloroform: isoamyl alcohol (0.5 ml) was added, vortexed and spun for 10 min at 12000 x g.

The upper phase 850 µl was removed and introduced into a clean eppendorf tube following which exactly 0.54 volumes of cold isopropanol were added. The mixture was centrifuged at 12000 x g for 20 s after visible pellets were washed 3 times in 70% ethanol. The pellets were resuspended in 50 µl TE (10mM Tris, 1mM EDTA pH7.0). To remove RNA, 1µl of RNase was added to the suspension and incubated at 37 °C for 30 min after which the DNA was stored at -20 for future use.

2.10 Multiplex PCR Serotyping and lineage classification of *Listeria monocytogenes*

Molecular serotyping was carried out according to the method of Doumith *et al.* (2004). Three reference strains and two environmental strains with known serotypes were used as positive control. Amplification was performed with Techne 312 thermocycler in a final volume of 50µl containing 1U of Taq polymerase(ABgene), 10ng DNA template, 0.2mM deoxyribonucleoside triphosphate (Promega) 2mM MgCl₂ (Abgene) and PCR Buffer (Abgene). The primer sets with sequences shown in Table 2.3 were added at the following concentration 1µM for *Imo0737*, *ORF2819*, and *ORF2110*; 1.5µM for *Imo1118* and 0.2 µM for *prs*.

PCR was performed with an initial denaturation step at 94 °C for 3 min followed by 35 cycles of 94 °C for 0.40 min, 53 °C for 1.15 min and 72 °C for 1.15 min and one final cycle of 72 °C for 7 min in a thermocycler (Techne 312). The reaction mixture (5 µl) was mixed with 2 µl of loading buffer and separated on a 2% agarose gel in TAE buffer. Staining with ethidium bromide enabled PCR products to be visible under UV light. After scoring the PCR products against a 100 bp ladder, serotypes were assigned to the strains based on the pattern of genes amplified.

2.11 Cell Hydrophobicity of *Listeria* cells grown in four different growth media.

The first part of the hydrophobicity tests was performed by the method of Rosenberg (1980). Cells were grown overnight in 5 ml of D10, BHI and D10 supplemented with duck meat (10:1) to 0.4-0.5 OD and harvested by centrifugating at 5000 x g. The cells were washed twice in PuM buffer (22.2 g K₂HPO₄.3H₂O, 7.26 g K₂H₂PO₄, 1.8 g Urea and 0.2 g MgSO₄) before re-suspending in 8mls of PuM buffer. Initial OD was taken at 400 nm after which 2 ml of suspended cells were transferred into 4 test tubes.

Various volumes (50, 100, 150 and 200 μ l) of the test hydrocarbon, n-octane were added. The test tubes were incubated at 37 °C for 10 minutes after which they were vortexed (Clifton Cyclone) for 120 s following which the test tubes were allowed to stand for 15 min. The light absorbance of 1ml of the aqueous phase was measured at 400 nm and results expressed as a percentage of cells recovered in aqueous phase relative to control.

Each experiment was performed in triplicate by using three independently prepared cultures.

A standard MATH assay as described by Briandet *et al.* (1999) was also carried out by using volumes 50-500 μ l of n-octane, hexadecane and chloroform. The tests were also carried out using different vortexing times, volumes and a different buffer (NaCl buffer). Results were obtained for percentage affinity of the cells to hydrocarbon by using the equation

$$\% \text{ Affinity} = 100 \times (1 - A/A_0)$$

Where A is the optical density at 400 nm of the bacteria suspension before mixing and A_0 is the absorbance after mixing.

2.12 Phase Contrast microscopy for *Listeria* cells grown in different media and vortexed with different solvents.

Cells of strains Lm 10403S were prepared as in Section 3.4. Harvested cells were grown overnight in BHI, MCDB 202, D10 and DJ+D10 at 37 °C with shaking. The cells grown in each media were resuspended in 2 ml of 150 mM NaCl buffer after which the cells were overlaid with 500 μ l of hexadecane, n-octane or chloroform and then vortexed for 120 s. After vortexing, the samples were left standing for 15 min to allow them to separate into aqueous and non aqueous layers.

Wet mounts of cells from each layer or phase were prepared by placing a drop on a clean glass slide then covered with a cover slip. These were viewed with the phase contrast (Hitchins *et al.*, 1968) microscope

(Nikon UK Ltd) using oil immersion objective (Phase ring 100 and 1000 x magnification).

2.13 Confocal microscopy for *Listeria* cells grown in different media.

Confocal microscopy has the added advantage of optical sectioning (Lawrence *et al*, 1991). Samples from different phases were viewed under the phase contrast microscope (Nikon, Japan) to determine cell distribution. Cells from only the non-aqueous phase were viewed under the confocal microscope (Bjorkov and Fiksdal, 2009) to determine how the cells attach to the solvent emulsion. Samples for confocal laser microscopy images were collected using a Nikon Eclipse Ti inverted Confocal microscope (Nikon UK Ltd) The equipment comprises lasers: Argon Ion 488 nm, Green Helium- Neon 543 nm, Blue diode 405 nm and is fitted with a C1 detector unit (3 PMT), a C1 transmitter detector unit (transmitted light), and the data collected and analysed with EZ-C1 control software. The samples were stained prior to imaging with Rhodamine B (excitation 561 nm and emission 567-650 nm).

2.14 Nigrosin capsule stain for cells grown in four different media.

The capsule stain was carried out as described by Kapale *et al*. (2003). A uniform film of *Listeria* culture was placed on a clean grease free slide, dried in air and fixed by heat. The film was covered with carbol fushin (diluted 1:3 with RO water) for 1 min, washed off with tap water and dried with blotting paper after which a drop of 10% Nigrosin (made with distilled water) was placed at one end of the film and spread evenly by drawing a film with the edge of the cover slip. The film was dried in air and observed with oil immersion objective.

2.15 Giemsa capsule stain for cells grown in D10 media

This was performed as described by Collins *et al*. (1995). The cells were fixed in absolute methanol for 3 min. Giemsa stain (BDH) was

poured on the slides and left until it was almost dried up before washing rapidly in water and then PBS. The slide was blotted and dried and then viewed under the light microscope with oil immersion at 100X magnification. Areas with pink stain for capsules or blue for bacteria cells were noted.

2.16 Scanning electron microscopy for *Listeria* cells grown in different media.

Scanning electron microscopy was performed as described by Cachat *et al.* (2008) Cells of *Listeria monocytogenes* and *Listeria innocua* grown overnight and harvested by centrifugation after which the cells were grown overnight in BHI, D10, D10 + D10 and MCDB202. Cells were harvested by spinning at 3000 x g for 15 min and then fixed in acetone (Fisher Scientific) for 1 hr. A drop of cell/acetone mixture was placed on the SEM stubs and allowed to dry. A drop of tetramethylsilane (Aldrich) was placed on the stub to enhance and complete the drying after which the stubs were coated with platinum by spurting (Polaron SC7640) for 90 s. The stubs were finally viewed under high vacuum with field emission gun SEM (Phillips XL 30). Purified capsular polymer was also subjected to the same treatment.

2.17 Extraction and purification capsular polymer from *Listeria monocytogenes*.

Glass beads (Stratech Scientific) were prepared as described by Troy (1973). The beads were soaked in 10% HCl overnight, rinsed off with RO water 6 times and then washed once in acetone (Fischer Scientific) before air drying under the fume cupboard. The extra polymeric capsular material was extracted and purified as described by Goto and Kunioka (1992), Kambourova *et al.* (2001) and Cachat *et al.* (2008).

The *Listeria monocytogenes* strain LM10403S was grown overnight in D10 media for two days and checked for capsule production with nigrosin stain. Glass beads (0.3 g) were added to an equal volume of

culture broth and ice (30mls in total) and vortexed for 2 min. The broth was decanted into centrifuge tubes and centrifuged at 15000 x g and 4 °C for 10 min after which the supernatant was collected. Four volumes of previously cooled ethanol cooled to -20 °C were added to the supernatant and left to precipitate for 24 h at -20°C.

The supernatant was then removed and the pellets dissolved in minimum amount of distilled water and Lyophilized (Telstar) under vacuum over night at -85 °C to get crude crystals. The crude crystals were re-suspended in 100 volumes of distilled water and centrifuged for 15 min at 20000 x g and 4 °C. The supernatant was dialyzed (Float-A-Lyzer®) overnight and lyophilized again to give pure crystals. This was also reconstituted at 5mg/ml.

To ensure that the pronase enzyme used for removing associated proteins are removed from capsular materials, the reconstituted pure crystals were filtered using a 100,000 mw sieve (Microcon™; Amicon USA). A 500 µl of the reconstituted material was centrifuged for 12 min at 500 g and lyophilized thereafter.

2.18 SDS - Polyacrylamide analysis of pure capsular crystals.

The bands of the capsular EPS were viewed by carrying out SDS-PAGE (Laemmli, 1970) as described by Kambourova (2001). The reconstituted capsule solution was treated with pronase (Boehringer Mannheim) before running on a 10% gel. Untreated samples served as control. The separating and stacking gel were polymerized for 40 mins by the addition of ammonium persulphate (Sigma) and N,N,N,N - tetramethylethylene - diamine (TEMED) (Sigma).

The gel was run in tank (Biorad) containing running buffer (Table2.3) at 200V for 3 hours using protein standards (Myosin, 198 KDa; β-Galactosidase, 115 KDa; Bovin serum albumin, 96 KDa; Ovalbumin, 53 KDa; Carbonic anhydrase, 37 KDa; Soybean trypsin inhibitor, 27 KDa; Lysozyme, 19 KDa and Aprotinin, 6 KDa; (Biorad)). The gel was

then stained in Coomassie brilliant blue, destained in destaining solution and stained with 0.5% methylene blue in 3% acetic acid before destaining with water. A similar gel was prepared and loaded with samples after which SDS-PAGE was carried out before staining the gel with silver stain.

Silver stain (Heukeshoven and Rudolf, 1985) was performed using the PlusOne Silver Staining Kit, Protein (GE Healthcare). After preparing the gel, it was fixed in the gel fixing solution for 60 min before sensitizing for 60 min and then washed four times for 15 min each time before adding the silver solution. After 60 min, the silver solution was removed, washed twice for 1 min and then developed for 4-6 min. When the bands reached the desired intensity, the gel was transferred to a stop solution and left for 60 min. The gel was finally transferred to the preservation solution and left for 60 min. All steps were performed with gentle shaking of the staining tray.

2.19 Sugar analysis of capsular polymer

Simple sugar analysis was carried out by UV analysis using the Sucrose/D-glucose/D-fructose kit (Boeringer Mannheim Cat No 716260) while an acid hydrolysis with 1 M H₂SO₄ at 100 °C was performed before analysing for complex sugars.

2.20 Amino acid analysis of capsular polymer

For amino acid analysis (Gilani and Sarwar, 2005), an amino acid analyser (Biochem 20 AA analyser; Pharmacia Biotech) with ion exchange column and detector was used. An approximately 300mg sample was placed in the refridgerator at 8 °C to cool for 2 h. After cooling 5 ml of chilled phenol was added to oxidize and kept for minimum 16 hours after which 0.84 g metabisulphite was added to the mixture before hydrolysing with 6 M HCl for 24 hours. The hydrolysate was adjusted to pH 2.20 using trisodium-citrate. The sample was then neutralized by 7.5 N NaOH solution before adjusting the sample again to pH 2.20. The mixture was rinsed 3 times with pH 2.20 tri-sodium

citrate buffer and 20 ml of the hydrolysate transferred to a centrifuge tube and centrifuged at 3000 x g for 2 minutes. The supernatant was passed through a 0.22 μm filter before loading onto the amino acid analyser. The amino acid was determined by ion exchange chromatography and reaction with ninhydrin using photometric detection at 570 nm.

2.21 Size exclusion chromatography coupled to multi-angle laser light scattering (SEC-MALLS)

This was performed as described by Morris *et al.* (2009). A couple of SEC columns TSK G6000PW and TSK G4000PW protected by similarly packed guard column (Tosoh Bioscience, Japan) was used to carry out analytical fractionation with on-line MALLS (Dawn Hellios, Wyatt Technology, U.S.A.) and refractive detectors (Vicostar II, Wyatt Technology, U.S.A.) The eluent (0.2M pH 4.3 acetate buffer) was pumped at 0.8ml min⁻¹ and a 100 μl volume was injected for the *Listeria* polymer and PGA. The absolute weight-average molar masses were calculated with ASTRA[®] software (Version 5.1.9.1, Wyatt Technology, U.S.A.) using the refractive index increment, $dn/dc = 0.185 \text{ ml g}^{-1}$.

2.22 Nuclear magnetic resonance (NMR) analysis of capsular polymer from *Listeria monocytogenes*.

Solid and liquid NMR was carried out for the *Listeria* polymer as using standard NMR methods (Solanky, 2005) as follows:-

2.22.1 Liquid NMR

The ¹³C NMR spectra were acquired on a Bruker Av 500 spectrometer as. A 30mg/ml or 5mg/ml was weighed and dissolved in deuterium water (4%). The sample was placed in NMR tubes up to the 4mm mark and placed inside the spinner of the spectrophotometer. The position of the spinner was adjusted to fit into a gauge. The sample was loaded on top of the magnet before acquiring and processing peaks by peak picking. The spectrum obtained was viewed on the visual display screen. Some of the dry crystals was dissolved in D₂O

(4%) at a concentration of 5mg/ml, placed in NMR tubes (Sigma) up to 4cm level and analysed for ¹H and ¹³C nuclear magnetic resonance

2.22.2 Solid State NMR: Cross Polarisation Magic Angle Spinning Nuclear Magnetic Resonance (CPMAS NMR)

The parameters used in these experiments are as follows. The Proton 90° pulse length was approximately 6.5 μsec. As stated previously, values of contact time of 1 and 5 msec were selected. Field strength of the proton and spin locking fields was approximately 40 KHz. For SPEMAS experiments, the decoupling power was varied, which was able to give a further insight into the rigidity of samples. Samples were packed into 7mm rotors and spun at speeds of 3.75 KHz. Reference ppm scales were provided, using the high field line of adamantane (29.5 ppm). Adamantane was run under identical conditions to the samples and at the same time to obviate effects of magnet drift. The ppm scale was felt to be accurate to approximately +/- 1.5 ppm.

For data processing, 8-16 K of data points were normally recorded with a time between points (dwell time) of 10 μseconds. 20 Hz of Lorentzian line broadening was then applied. The data set was Fourier transformed and phased with 0 and 1st order corrections. It was suspected that a gain stage in the preamplifier, not under control of the spectrometer gain setting, was experiencing soft overload, as there was a pronounced baseline dip under the major peaks. This was corrected by baseline fitting routines which were consistently applied to all spectra; however it was felt that this was a source of potential error in the subsequent fitting routines.

2.23 Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy analysis

Standard FTIR was performed as carried out by Berthomieu and Hienerwadel (2009). Spectra were recorded using a Bruker (Tensor 27, Germany) fitted with a deuterated triglyceride sulphate (DTGS) detector. FTIR was connected to an air dryer (Balston, US) to purge the instrument of water vapour and carbon dioxide. Diamond ATR

spectra of the dry powders were recorded using the diamond golden gate accessory (Specac). A small amount of the sample material (powder) was placed onto the diamond and contact with the diamond achieved by pressing the sample down using the anvil. Data were collected as interferograms at 4 cm^{-1} resolution, where 128 interferograms were collected and co-added. Spectra were obtained by ratioing against a background spectrum of air. For clarity, spectra are presented offset.

2.24 X-ray Diffraction analysis

Standard X-ray diffraction as performed by Langan *et al.* (2001) was carried out. X-ray diffractograms were recorded on powdered samples as supplied, for values 2θ between 4 and 38 degrees at 0.1 degree intervals with a scanning time of 6 s per interval, using a Bruker D5005 (Bruker AXS, UK) diffractometer at 20 °C. The diffractometer was equipped with a copper tube operating at 40 kV and 40 mA producing Cu K alpha radiation of 1.5418 Å wavelength. Several Samples were also run on a Bruker D8 operating at 40 kV and 40 mA in focusing transmission geometry configured with a LynxEye detector. Phase analysis and identification of crystalline forms were achieved using Bruker Diffrac^{plus} and Eva software coupled to X-ray identification databases.

2.25 Statistical analysis of data

Data analysis was performed using the Data Analysis Tool Pack in Microsoft® Excel 2007. All analysis was based on 95% confidence limit.

CHAPTER 3 RESULTS:

**The effect of low nutrients on growth, flocculation
and surface charge of *Listeria* cells**

3.1 Introduction

It has been pointed out that the contamination of surfaces by pathogens is a major concern in the food industry because the biofilms that form result in persistent contamination of food factories, and this can lead to contamination of products and therefore increased risks to public health (Van houdt and Michiels, 2010). Cases of *L. monocytogenes* have increased throughout Europe and in the last few years the persistence of *L. monocytogenes* in food processing environments has been recognised as a source of contamination (Takahashi *et al.*, 2010). Bacterial adhesion is a crucial step in the biofilm formation process (Chmielewski and Frank (2006) and *Listeria* is able to attach to surfaces and form biofilms (Little *et al.*, 2009) which consist of viable and non-viable microorganisms embedded in EPS anchored to a surface.

It has been reported widely that *Listeria* is able to withstand adverse conditions which include low nutrient availability, high or low pH and temperature (Ghandi and Chikindas, 2007; Gabriel and Nakano, 2010; Zhang^a *et al.*, 2010), and to do this it undergoes a period of adaptation. Rodrigues *et al.* (2009) studied the effect of batch and fed-batch growth on *Listeria* cells and found that different growth modes significantly influenced attachment of *L. monocytogenes* to abiotic surfaces as well as resulting in changes in metabolic activities within the cells. Since in most food processing environment, there are periods of low and high availability of nutrients, it is important to determine how different growth conditions affect attachment of *L. monocytogenes* cells to surfaces.

Recently, there has been an increase in the reporting of *Listeria* in duck meat products. In the UK Li (2004) used molecular typing methods to trace the source of *L. monocytogenes* contamination in a bakery and found that a particular problem was associated with duck

meat product. Chipilev *et al.* (2010) reported an increase in distribution of *L. monocytogenes* from slaughtering to vacuum packing of duck breast and liver while the recall of duck leg and sausage products was due to possible *Listeria* contamination (Anon^c, 2009). Similarly Karakolev (2003) analysed 112 samples of goose meat and liver samples and isolated *L. monocytogenes* in 35% and 4.8% of the samples, respectively, indicating that the meat itself was the source of contamination rather than infected birds being processed.

Therefore, the aim of work described in this Chapter was to determine how different growth conditions or nutrient availability affect the growth and surface properties of *L. monocytogenes*. It was also planned to determine if the nutrients supplied from duck meat resulted in increased growth or attachment.

3.2 Media preparation and initial growth of *Listeria* cells in different media

The four different media used for the study included a rich media (BHI), a chemically defined media (D10), and a commercially available chemically defined media (MCDB 202) that had previously been used in studies of *Listeria* biofilms (Chavant *et al.*, 2002). In addition the effect of supplementing D10 media with duck juice was investigated.

The BHI medium was prepared according to manufacturing instructions while the chemically defined minimal medium MCDB 202 was prepared as described by (Chavant *et al.*, 2002; see Section 2.1.5). The D10 media consists of 18 different compounds which are known to support *L. monocytogenes* was assembled as described by Trivet and Meyer (1971; see Section 2.1.3 for more details).

The fourth media was prepared by adding duck meat extract to D10 media. Duck meat juice was prepared by extracting juice from duck meat fillets. Briefly, the fatty skin was removed and then the meat cut into small pieces, boiled for 20 min, and then the cooked meat stomached in sterile distilled water. The mixture was filtered and then sterilized by autoclaving (see Section 2.1.4).

Since the nutrient contribution of duck meat juice to growth of *L. monocytogenes* is unknown, the first thing to be determined was the optimum concentration of the duck meat juice that would sustain growth of the organism. The well studied *Listeria* strain Lm 10403S was used as a model laboratory strain and was grown in D10 supplemented with three different concentrations of duck meat juice prepared by mixing the media at ratios of 1:1, 1:5 and 1:10 with duck meat juice. Cells were grown at 37 °C with shaking and growth monitored over time by monitoring at OD_{600nm}. As control the bacteria were also grown in D10 without duck meat juice and in BHI, to compare results gained with media known to produce a high growth rate. Growth rate was calculated using the formula

$$\mu = \log_{10} (N - N_0) 2.303 / t - t_0$$

Where μ = growth rate constant

N_0 = number of cells at the beginning of log phase

N = number of cells at some later time

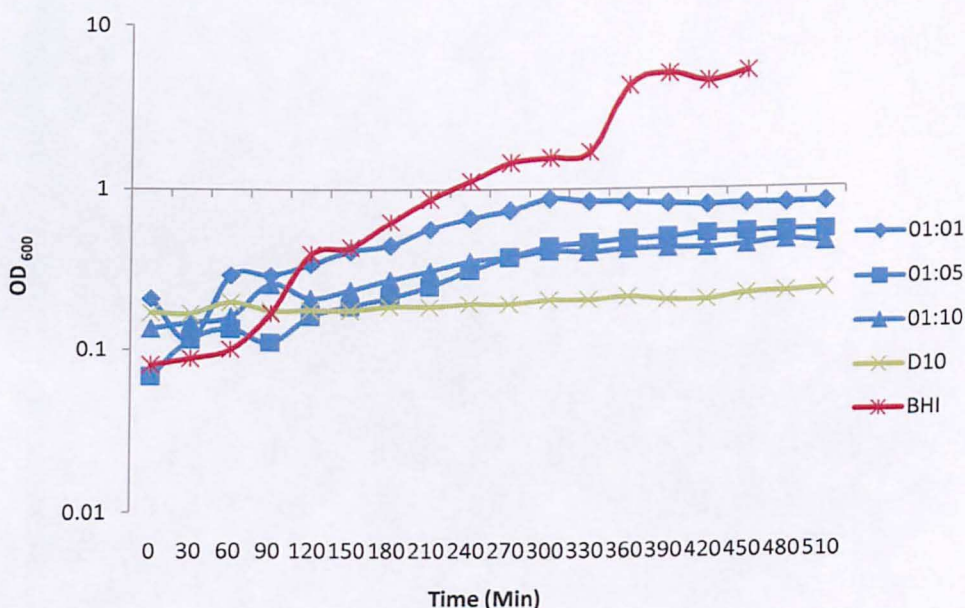
t_0 = time at the beginning of log phase

t = time at a later time

Expectedly, cells grown in BHI (Fig. 3.1) had the highest growth rate (1.04 h⁻¹) while cells grown in D10 had the lowest growth rate (0.14 h⁻¹). The cells grown in the D10 supplemented with duck meat juice all produced different enhanced growth rates of (0.32, 0.39 and 0.21 h⁻¹)

for cells grown in 1:1, 1:5 and 1:10 DJ+D10 mix, respectively. Hence the D10 with 10 % duck meat juice supplement (DJ+D10) was used for further studies in order to monitor growth easily since the higher levels of duck juice did not further enhance growth and made the broth more opaque and therefore more difficult to accurately monitor growth.

Figure 3.1 Preliminary assessment of growth of *L. monocytogenes* Lm 10403S in different media



Lm 10403S was inoculated into different media and grown at 37 °C, with shaking. Media used were BHI (—*), D10 (—x), and D10 supplemented with different ratios of duck meat juice (1:1, —◇; 1:5, —□; and 1:10, —△).

3.3 Molecular Serotyping of *Listeria* Strains used

For this work, in addition to the standard laboratory strains, it was also planned to use some environmental strains of *L. monocytogenes* that were isolated from a bakery where a particular problem had been identified with duck meat products (Li, 2004). However, while the serotypes of the reference strains were known, the serotypes of these environmental strains were unknown.

Hence molecular serotyping was performed to establish the serotypes of the strains that would be used in the study. Molecular serotyping is now a widely accepted typing method for *L. monocytogenes* and permits differentiation between important food-borne serotypes (1/2a, 1/2b, and 4b; Boruki and Call, 2003).

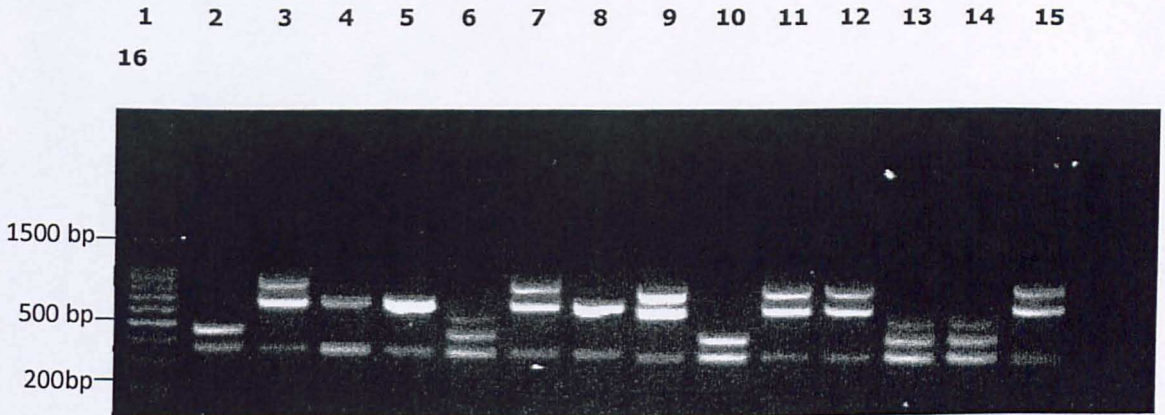
To type the strains, DNA was extracted from reference strains and the uncharacterised factory isolates as described by Pitcher *et al.* (1989; see Section 2.9). Molecular serotyping was carried out by PCR according to the method of Doumith *et al.* (2004). The primer sets used with sequences are shown in Table 2.6.1 and included *Imo0737*, *ORF2819*, *ORF2110*, *Imo1118* and *prs*. (see Section 2.10). Nine factory isolates with unknown serotypes and three reference strains and two environmental strains with known serotypes were used as positive controls.

Figure 3.2 shows the results of the PCR serotyping. All the strains produced a PCR product corresponding to a fragment of *prs* which is a gene located at one end of the main virulence gene operon. In addition 4b strains produced PCR products from *ORF2110* and *ORF2819* (597 and 471 bp, respectively) while serotype 1/2a strains produced PCR products of 691 bp corresponding to *Imo 0737*. All serotype 1/2c strains produced PCR products of 691 bp from *Imo0733*

and 906 bp from *Imo1118* and strains of serotype 1/2b produced a PCR product of 471 bp from *ORF2819*. A summary of the results gained is shown in Table 3.1.

The main finding was that 4 out of the 9 strains tested were of the important food-borne strains (1/2a, 1/2b, and 4b) most often associated with large out breaks of food-borne Listeriosis (Ward, 2004), and since these organisms were found to be endemic in the factory, they may have been transferred from the food processing environment onto the finished food products. Importantly for this study they represented a range of different serotypes of factory adapted strains and therefore provided good test strains which could be compared with the more well studied but lab-adapted strains often used in studies of *Listeria*.

Figure 3.2. Molecular serotyping of *Listeria* strains



Multiplex PCR Serotyping of *L. monocytogenes* isolated from an environment associated with duck meat *Listeria* contamination. PCR was performed with an initial denaturation step at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 0.40 min, annealing at 53 °C for 1.15 min and extension at 72 °C for 1.15 min and one final extension cycle of 72 °C for 7 min. The PCR products were separated on 2.0 % agarose gel. After sizing the PCR products against a 100 bp ladder, serotypes were assigned to the strains based on the pattern of genes amplified. Lane1, 100 bp ladder; Lane2 = Lm4 (1/2b), 3 = Lm2 (1/2c), 4 = LmEGD-e, 5 = Lm10403S, 6 = Lm23074, 7 = Lm0006-0305 (1/2c), 8 = Lm000622-0305 (1/2a), 9= Lm000630-0305 (1/2c), 10= Lm00054-0305(1/2b), 11=Lm000636-0305(1/2c),12=Lm000480305 (1/2c), 13= Lm000101-0305 (4b), 14= Lm00064-0305 (4b), 15= Lm00006-0305 (1/2c), 16=Blank (No DNA).

Table 3.1: Molecular Serotyping of *Listeria monocytogenes* from a Bakery in UK using multiplex PCR.

| Strain | Serotype* | Multiplex PCR Fragment Amplification | | | | | Multiplex PCR Serovar group Classification |
|----------------|-----------|--------------------------------------|----------------|---------|---------|------------|--|
| | | <i>lmo1118</i> | <i>lmo0737</i> | ORF2110 | ORF2819 | <i>prs</i> | |
| | | (906bp) | (691bp) | (597bp) | (471bp) | (370bp) | |
| Lm 23074 | 4b | – | – | + | + | + | 4b |
| Lm 4 | 1/2b | – | – | – | + | + | 1/2b |
| Lm 27 | 1/2c | + | + | – | – | + | 1/2c |
| Lm EGD | 1/2a | – | + | – | – | + | 1/2a |
| Lm 10403S | 1/2a | – | + | – | – | + | 1/2a |
| Lm 00101-0305 | Unknown | – | – | + | + | + | 4b |
| Lm 00048-0305 | Unknown | + | + | – | – | + | 1/2c |
| Lm 0054-0305 | Unknown | – | – | – | + | + | 1/2b |
| Lm 00064-0305 | Unknown | – | – | + | + | + | 4b |
| Lm 00630-0305 | Unknown | + | + | – | – | + | 1/2c |
| Lm 000622-0305 | Unknown | – | + | – | – | + | 1/2a |
| Lm 00636-0305 | Unknown | + | + | – | – | + | 1/2c |
| Lm 0006-0305 | Unknown | + | + | – | – | + | 1/2c |
| Lm 00006-0305 | Unknown | + | + | – | – | + | 1/2c |

***Strains of known serotypes were used as positive controls for the PCR assay**

3.4 Growth of *Listeria* strains in four different media

The growth of *Listeria* in 4 different media was performed in order to determine the effect of different nutrient levels on the growth of the bacteria and to investigate how the organisms respond to the presence of the duck juice supplement. To generate starter cells for this study, two reference strains Lm 10403S (serotype 1/2a), Lm 23074 (serotype 4b) and three environmental strains Lm 00054-0305 (serotype 1/2b), Lm 000622-0305 (serotype 1/2a) and Lm 000101-0305 (serotype 4b) were grown in BHI at 37 °C overnight with shaking.

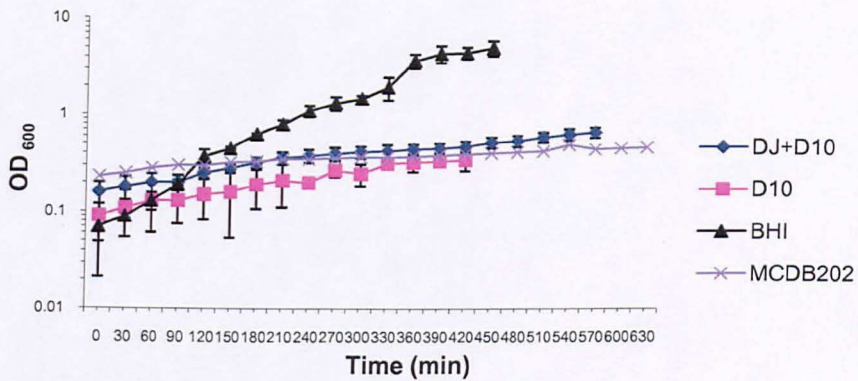
The strains chosen represent the main serotypes associated with *Listeria* outbreaks of Listeriosis. The cells were harvested by centrifuging at 3000 x g and the pellet was washed twice with PBS using the same centrifugation conditions. The washed cells were then inoculated into 100 ml of the four different media in a 250 ml conical flask and grown at 37 °C with shaking. This method of generating starter cells was used because of the very slow growth rates observed in D10, so that it was difficult to generate sufficient cell mass to prepare inoculums and the slow growth rate meant that cells cultures in different media were in different phases of growth after overnight incubation. Hence using this method also ensured that cells introduced into the different media were of similar age and physiological state. Growth rate was monitored by measuring OD_{600nm} over time and growth rate was calculated as in described in Section 3.2.

The growth curves are shown in Figure 3.3a-e. Curves were plotted for longer times in the minimal media because it took longer to get to the exponential phase. As expected in each case cells grown in BHI had the highest growth rate followed by those grown in DJ+D10 showing that there was increase in growth when duck juice was added to the minimal media. Since the same duck juice extract was used for all the

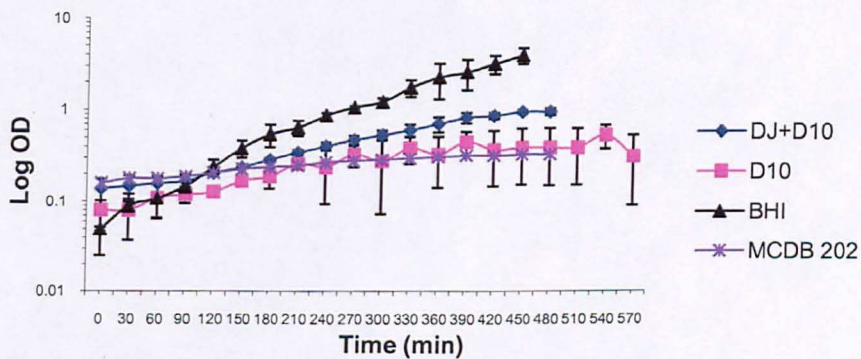
strains, it suggests that the growth rate of *Listeria* strains in media supplemented with duck juice extract was different.

Figure 3.3 Growth of *Listeria* cells in four different media

a) Lm 23074

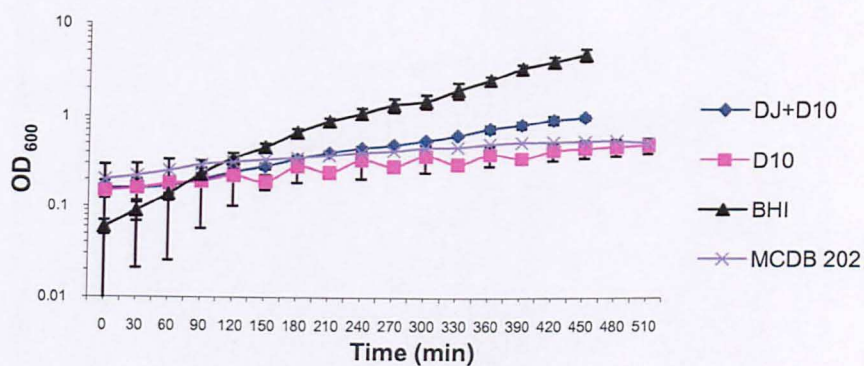


b) Lm 10403S

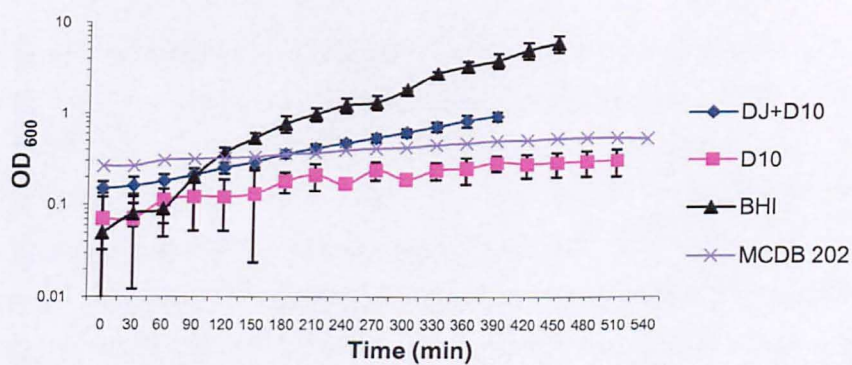


Cells were grown with shaking at 37 °C in BHI (▲), D10 (■), DJ+D10 (◆), and MCDB 202 (×). Strains used were Lm 23074 (panel a) and Lm 10403S (panel b). Error bars represent 2 standard errors.

c) Lm 000622-0305

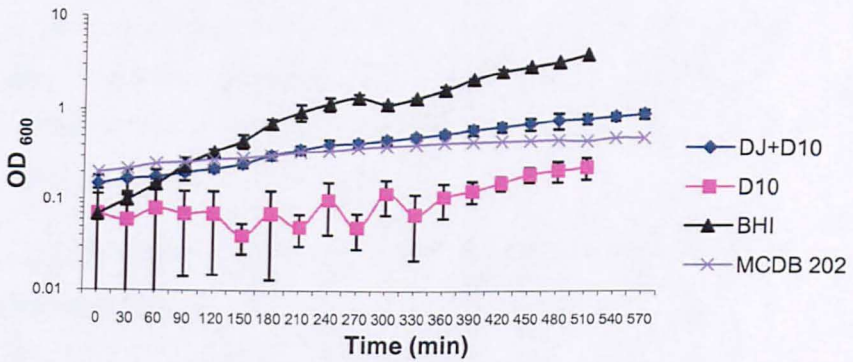


d) Lm 000101-0305



Cells were grown with shaking at 37°C in BHI (▲) D10 (■), DJ+D10 (◆), and MCDB 202 (×). Panel (c) Lm 000622-0305 and (d) Lm 000101-0305. Error bars represent 2 standard errors.

e) Lm 00054-0305



Cells were grown with shaking at 37°C in BHI (▲) D10 (■), DJ+D10 (◆), and MCDB 202 (×). Panel (e) Lm 00054-0305 (serotype 1/2b). Error bars represent 2 standard errors.

Analysis of variance (ANOVA) carried out on the means of the growth rate for cells grown in the four different media (BHI, D10, DJ+D10 and MCDB 202) showed that there were significant differences ($p < 0.001$) when the growth rate in four different media was compared and not significant when BHI was excluded from the set ($p > 0.05$). In all cases the duck juice did not increase the growth rate much but seem to lead to a higher final cell mass which suggests that some specific nutrients may be limiting in D10 alone.

The growth of *L. monocytogenes* in different media show that the organism can grow in wide range of nutrient limiting condition (Shin *et al.*, 2010). Pan *et al.* (2010) investigated the synergistic effect of glucose and temperature on growth and found that *Listeria* with serotype 1/2a formed higher density biofilms than serotype 4b despite the fact that the serotype 4b had more growth.

Rodrigues *et al.* (2009) studied the effect of batch and fed batch growth and found that different growth modes significantly influence *L. monocytogenes* attachment on abiotic surfaces as well as metabolic activities within the cells. Tresse *et al.* (2006) found that the physicochemical properties of the cell surface can affect bacterial auto aggregation and co-aggregation abilities. Hence this aspect of the effect of the different growth media on the ability of cells to persist in factory environments is of interest.

Table 3.2 Growth rate of *L. monocytogenes* strains in different media

| Strain/serotype | Growth rate (h ⁻¹) | | | |
|----------------------|--------------------------------|---------|------|------|
| | BHI | DJ+ D10 | MCDB | D10 |
| Lm 10403S (1/2a) | 2.53 | 0.31 | 0.30 | 0.29 |
| Lm 23074 (4b) | 1.37 | 0.12 | 0.23 | 0.16 |
| Lm 000622-0305(1/2a) | 3.23 | 0.32 | 0.25 | 0.16 |
| Lm 000101-0305(4b) | 2.71 | 0.29 | 0.22 | 0.14 |
| Lm 00054-0305(1/2b) | 2.35 | 0.17 | 0.22 | 0.20 |

Growth rate of *Listeria* strains in different media. Cells were grown in 100 ml broth with shaking at 37 °C and OD_{600nm} taken at intervals. In all broth used, strain1/2a had the highest growth rate. Cultures were prepared from 3 independent cultures.

3.5 Flocculation of cells grown in Different media

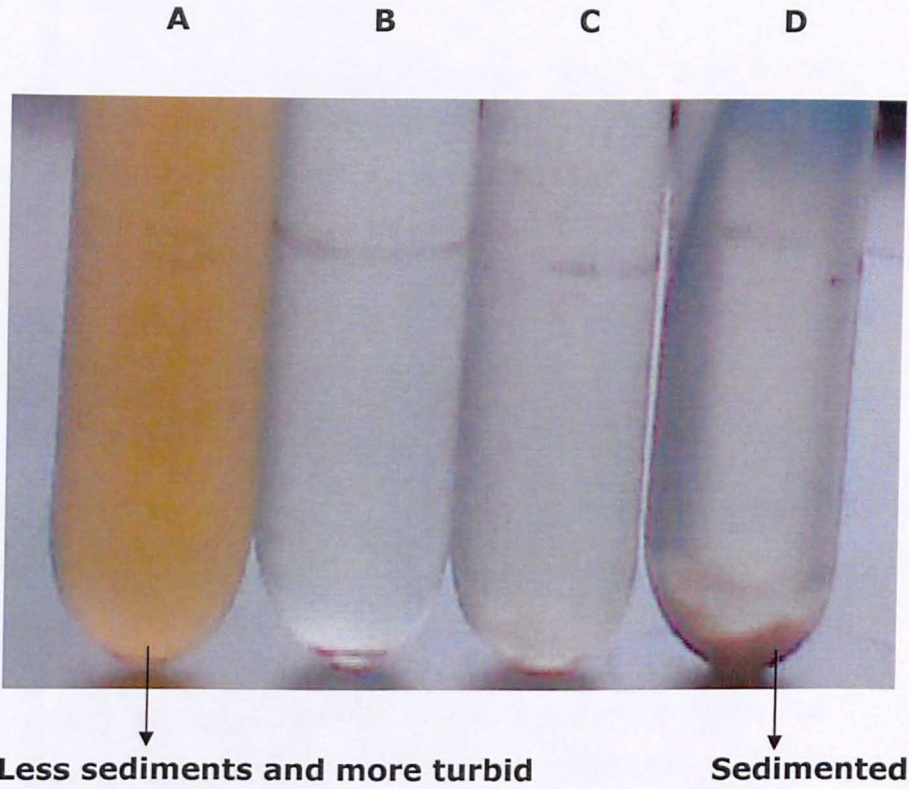
Palmer and White (1997) and O' Toole *et al.* (2000) reported that planktonic cells would need to aggregate before attachment and formation of biofilms and this is achieved by cell to surface and cell to cell attachment. During the growth experiments in Section 3.4, some differences in cell sedimentation were observed when the cells used for the inocula were resuspended in different media and this phenomenon was investigated further.

In order to determine how cells flocculate in different media, cells were grown and harvested as described in Section 3.4. Cells harvested from 10 ml of broth were transferred into 10 ml of the different broth (BHI, D10, D10+DJ, MCDB 202) in a test tube and were grown overnight with shaking at 37 °C after which the tubes were left to stand on the bench for one minute and the distribution of the cells in the media recorded. Further observations were made after 72 h incubation at 37 °C.

When the cells were grown in the defined media with shaking in the test tubes, they were observed to flocculate and sediment at the bottom of the tube, with the most sedimentation occurring when cells were grown in MCDB 202 medium (Fig. 3.4). The cell sediments formed were loose and were easily dispersed when the cells were vortexed. Cells appeared more loosely aggregated when grown in MCDB 202 compared to cells grown in D10. In contrast the cells grown in the other media produced more homogeneous cell suspensions but cell sedimentation was apparent after 72 h incubation. When the media was decanted from the test tube after 72 h, the sedimented cells at the bottom of the test tube appeared more slimy and sticky, with those grown in D10 medium being observed to have the slimiest nature.

An inspection of the constituents of the MCDB 202 medium (see Section 2.1.5) revealed that it has a slightly higher NaCl concentration than BHI (0.7% vs 0.5%). Takeda *et al.* (1992) showed that salts like NaCl, CaCl₂, Al(SO₄)₃ and FeCl₃ stimulated flocculating activity of a protein bioflocculant produced by the, mycolic acid-containing bacterium *Norcadia amarae*. Hence it was possible that this increased flocculation being seen was due to a change in the surface proteins of the *Listeria* cells. Although now an old observation, Mcgregor and Finn (1969) listed the factors that affect flocculation to include bacterial genus (perhaps now not an unexpected finding given the discovery of the genetic relatedness of different bacterial groups; Sallen, 1996), suspending medium, temperature, physiological age and surface shear. Also listed was naturally occurring polymers released in bacteria suspension which may increase or decrease the flocculants required for cellular aggregation. Hence there are a variety of different changes in the cell surface of *Listeria* that could result in the enhanced sedimentation of the cells grown in MCDB 202 and D10 media.

Figure 3.4 Flocculation and sedimentation of *Listeria* cells



cells

Overnight cultures of Lm1040S cells grown in BHI (A), D10 (B), D10 + DJ (C) and MCDB 202 (D). Cells were grown overnight with shaking at 37 °C after which the test tubes were left to stand for 1 min. Cells in MCDB 202 sedimented quickly while cells in BHI remained turbid throughout the tube.

3.6 Zeta potential analysis of *Listeria* cells grown in different media

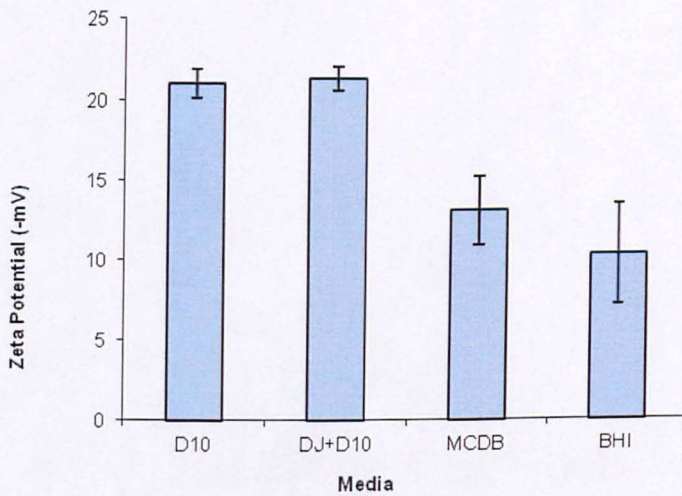
According to Singleton and Sainsbury (1987), zeta potential is the electrical potential at the surface of shear i.e. the surface of the cell or particle which tends to move when an electrical field is applied. Also it is a determinant of the electrophoretic migration rate of a cell. The analysis was performed to determine the nature of cell surface charge for cells grown in different broth cultures and to determine if the different growth conditions had any effect on the cell surface charge.

Cells of strain Lm 10403S were grown and harvested as described in Section 3.4. The cells were then inoculated into the four different media and grown with shaking at 37 °C overnight. A sample (5 µl) of the cells grown in each media was placed on the reader of the zeta potential instrument (Berkman Coulter Version 1.34) and readings from three separately prepared cultures were recorded. All the cells were found to be negatively charged (Fig. 3.5). It was found that cells grown in D10 and DJ+D10 media had the highest negative surface charge, followed by cells grown in MCDB 202. The cells grown in BHI had the least surface charge. Interestingly supplementing the media with duck juice did not alter the surface charge of the cells, suggesting that limited nutrients were not causing the difference in cell charge, rather some other component of the media.

This suggests that the media used may have an impact on the cell surface charge of the cells and may affect electrostatic interactions between *Listeria* cells. The flocculation results in Section 3.5 showed that cells flocculated most in minimal media and these cells are now known to be more negatively charged than those grown in BHI media. Briandet *et al.* (1999) established a link between electrostatic interactions and microbial adhesion to hydrocarbons. They reported that regardless of medium used to cultivate *L. monocytogenes* Scott A,

the cells always had affinity for electron acceptor solvents than non-polar solvents which shows that the organism is an electron donor.

Figure 3.5 Zeta potential analyses of *Listeria* cells



Zeta potential measurements of the broth culture of Lm 10403S cells grown in D10, D10+DJ, MCDB 202 and BHI broths. Results represent average of three samples and error bars represent 2 standard deviations. Measurements were obtained from 3 independent cultures.

3.7 Conclusions

The major serotypes involved in food-borne Listeriosis (1/2a, 1/2b and 4b) were found to be endemic in the factory environment and serotype 1/2a grew fastest in different media used.

- Cells grown in minimal media flocculated more than cells grown in rich media.
- Supplementing the D10 minimal media with DJ+D10 increased growth rate of the cells but its addition did not alter surface charge of the cells.

CHAPTER 4 RESULTS:

Effect of Low Nutrients on Cell Hydrophobicity

4.1 Introduction

In the previous Chapter the cells grown in minimal media were seen to flocculate and to have an altered surface charge. To determine if growth in the different media also affected cell hydrophobicity, the microbial attachment to hydrocarbons (MATH) assay was chosen because it is without any doubt the most widely used method to determine microbial hydrophobicity (Van de Mei and Busher, 1998).

The experiment was performed as described by Rosenberg (1980). To generate starter cells, different strains were inoculated in 10 ml of BHI broth and prepared as described in Section 3.4. Again, two reference strains Lm 10403S (serotype 1/2a), Lm 23074 (serotype 4b) and three environmental strains Lm 00054-0305 (serotype 1/2b), Lm 000622-0305 (serotype 1/2a) and Lm 000101-0305 (serotype 4b) were used. These strains were used because they represent the major serotypes involved in Listeriosis and we also wished to establish if the reference strains behaved differently to the environmental strains.

The prepared cells were inoculated into three different media (BHI, D10, and DJ+D10; see Section 3.4) and were grown overnight before harvesting by centrifugation at 3000 x g and re-suspending in 2 ml of the buffer described by Rosenberg *et al.* (1980) containing potassium urea magnesium (PuM) (Section 2.5) after which the optical density of the suspension was measured at 400 nm. An over lay of different volumes of the hydrocarbon n-octane (50-200 μ l) was added to the PuM suspension and then the sample vortexed for 15 s after which the mixture was allowed to settle for 15 s. After settling a sample of the aqueous layer was taken and the OD_{400nm} was determined. The hydrophobicity of the sample was determined by calculating the percentage affinity of the cells to the solvent using the equation:-

$$\% \text{ Affinity} = 100[1 - (A \times A_0^{-1})]$$

Where A_0 is the OD_{400nm} of the bacteria suspension before mixing with hydrocarbon and A is that after mixing. Each analysis was performed in triplicate using three independently prepared cultures.

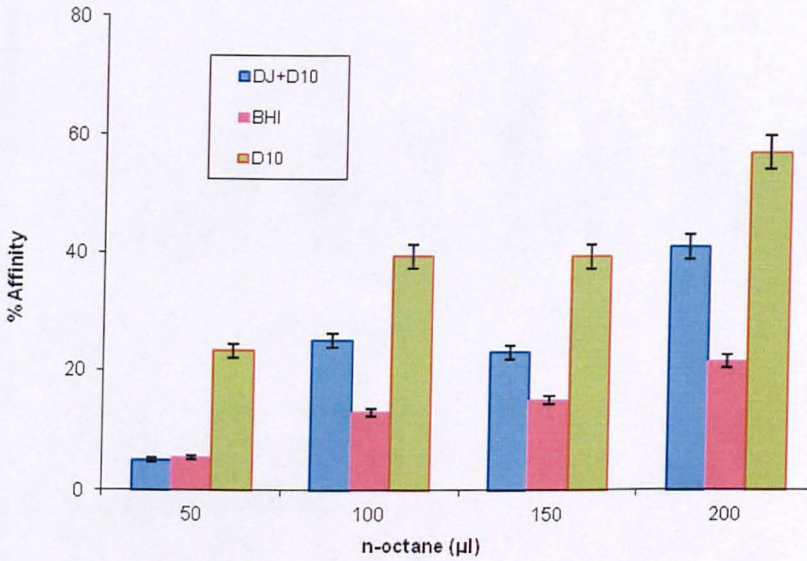
The results are presented in Figure 4.1. For all strains, cells grown in D10 or DJ+D10 generally had a higher affinity for the hydrocarbon than cells grown in BHI showing that growth rate was not a key factor in determining affinity as in Chapter 3, it was shown that cells grown in BHI had the highest growth rate in all samples. Interestingly it was also seen that cells grown in BHI had the lowest level of flocculation and now showed the least hydrophobicity when solvent affinity experiments were carried out which might explain the lower level of flocculation of the cells in the culture media.

However, the degree of difference in affinity detected between the D10/D10+DJ samples and BHI varied depending on the volume of solvents used. For instance at 50 μ l of solvent, no difference in hydrophobicity was seen between D10+DJ and BHI samples for some strains (Fig. 4.1a), but at all volumes above this BHI samples are clearly less hydrophobic for all strains (Fig. 4.1 a-e). Hence higher solvent volumes may be required to reach an end point of the partitioning assay for these cells.

There is a lot of debate in literature concerning the actual factors that could affect hydrophobicity, surface properties and attachment of *Listeria* to the hydrocarbons used in the MATH assay. These factors include electrostatic interactions (Geertsema-Doornbusch *et al.* 1993) between cell surface molecules and the solvent, Lewis acid-base interactions (Bellon-fontaine *et al.* 1996) and surface physicochemical properties (Chavant *et al.* 2002). Hence the experimental design was expanded to take some of these factors into consideration.

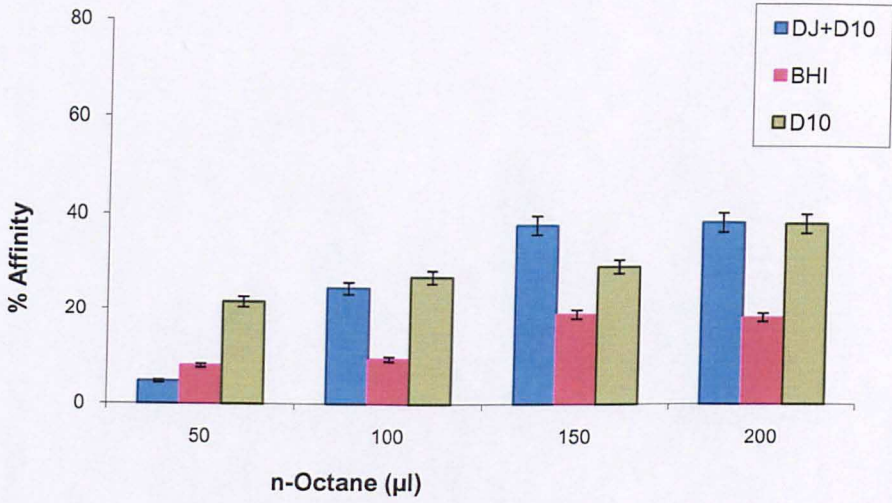
Figure 4.1 Cell hydrophobicity of *L. monocytogenes* in PuM Buffer using n-octane solvent

a) Lm10403S

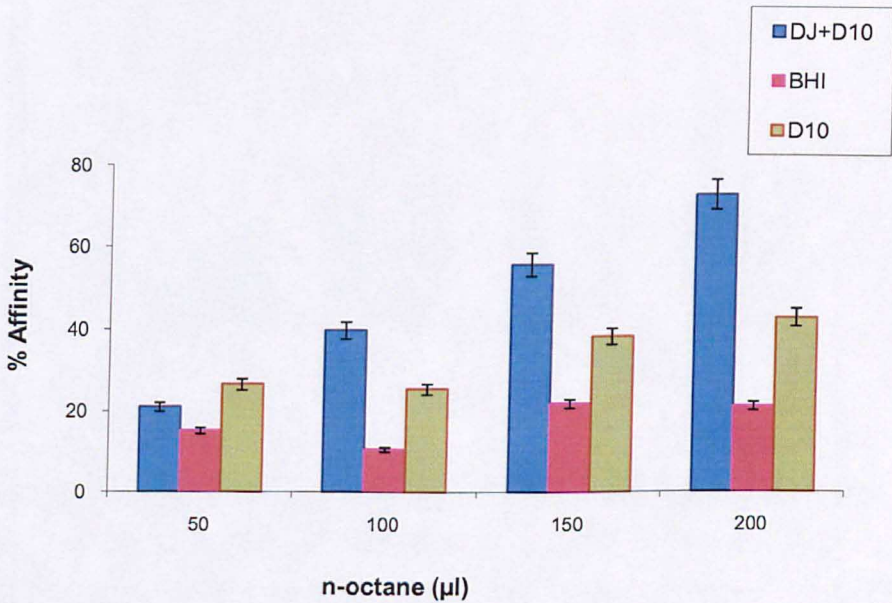


Cell hydrophobicity of Lm 10403S in PuM buffer after growth in D10 (■) DJ+D10 (■) and BHI (■) broths. Results represent triplicate independently prepared samples. Error bars represent 2 standard errors.

b) Lm23074

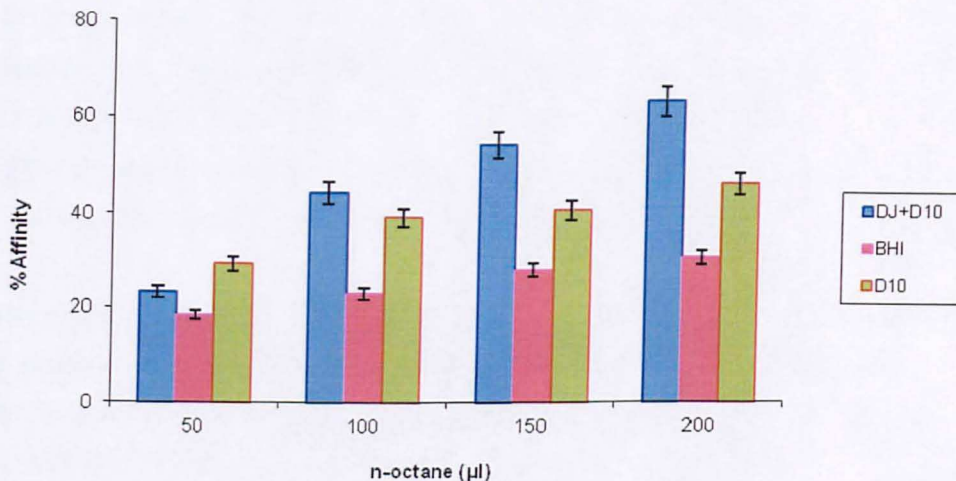


c) Lm 00054-0305

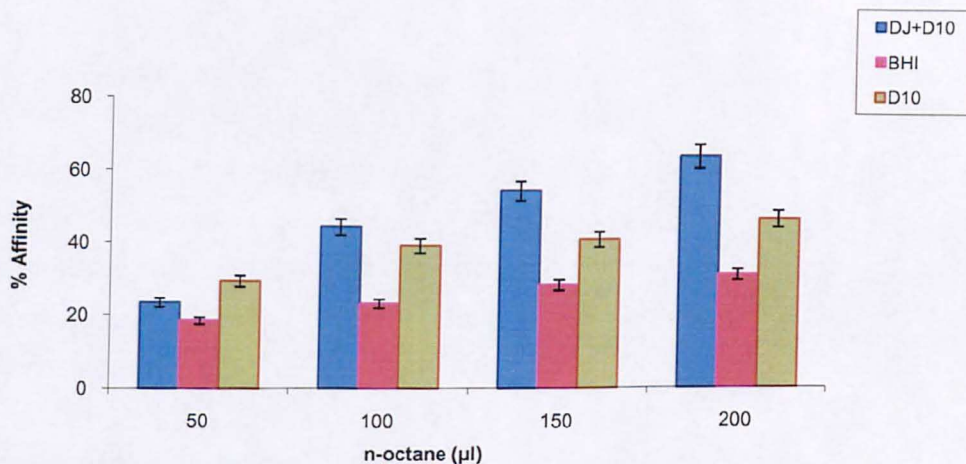


Cell hydrophobicity of Lm 23074 (b) and Lm 00054-0305 (c) in PuM buffer after growth in D10 (■) DJ+D10 (■) and BHI (■) broths. Results represent triplicate independently prepared samples. Error bars represent 2 standard errors.

d) Lm 000622-0305



e) Lm 000101-0305



Cell hydrophobicity of Lm 000622-0305 (d) and Lm 000101-0305 (e) with PuM Buffer after growth in D10 (■) DJ+D10 (■) and BHI (■) broths. Results represent triplicate independently prepared samples. Error bars represent 2 standard errors.

4.2 Effect of assay conditions and solvents on Hydrophobicity of *L. monocytogenes*

Since the end point was not reached in the volume range of 50-200 μ l in Section 4.1, the experiment was modified based on the work of the methods of Briandet *et al.* (1999) and Bellon-Fontaine *et al.* (1996). Specifically more solvents were used to measure the hydrophobicity of the cells and the buffer used to resuspend the cells was also changed.

Briandet *et al.* (1999) determined the variations in hydrophobicities and electron donor and acceptor characteristics of *L. monocytogenes* Scott A when the organism was grown in different growth media and temperatures. They used BHI and Trypticase soy/yeast extract (TYSE) and then supplemented TYSE with glucose or lactic acid. Furthermore, four solvents with different electrostatic properties, namely chloroform, hexadecane, ethyl acetate and decane, were used.

Also, they used a NaCl buffer instead of PuM buffer because the buffer has high concentration of electrolyte which can prevent charge interference by masking cell charge, especially because some solvent droplets, especially hexadecane, become negatively charged in aqueous suspensions and if charge on the cell surface is masked then a different interaction with the solvent will result (Geertsema-Doornbush *et al.* 1993).

As in Chapter 3, different strains were inoculated in 10 ml of BHI broth to generate starter cells. In addition to cells used in previous experiment (i.e. Lm 10403S, Lm 23074, Lm 00054-0305, Lm 000622-0305 and Lm 000101-0305), more strains were added to include widely studied strain Lm EGD-e which has a completely sequenced genome and a large body of biochemical, functional and genetic data available (Zameer *et al.* 2010). Another reference strain Lm LO28 (serotype 2c) used by Chavant *et al.* (2002) in a hydrophobicity study and its environment equivalent strain Lm 00048-0305 (serotype 2c) were included for comparison with previously published results. These

strains were added to provide a broader spectrum of strains for the study.

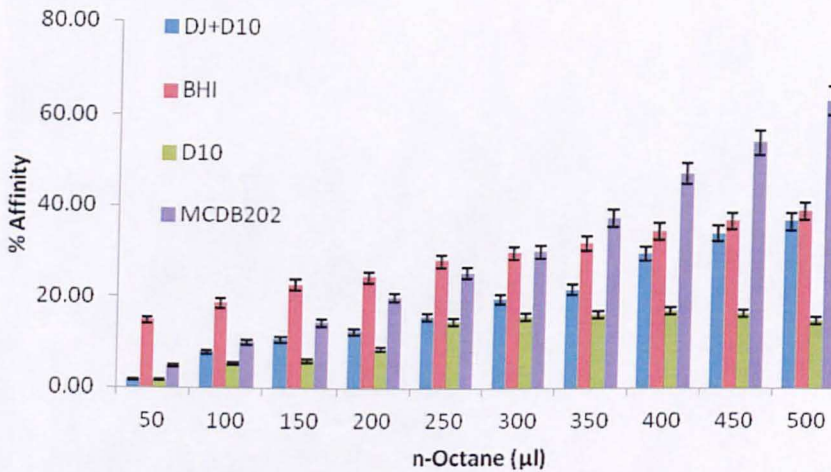
After overnight growth, cells were inoculated in four different media (BHI, D10, MCDB 202 and DJ+D10) and harvested by centrifugation at 3000 x g before suspending in 2 ml of 150 mM NaCl then the cell suspensions overlaid with chloroform, hexadecane or n-octane. Then the OD was measured at 400 nm both before and after the samples were vortexed and after being allowed to stand for the different phases to separate.

The results of cell hydrophobicity assays of different strains carried out after growing cells in different media and overlaid with n-octane and vortexed in 150 mM NaCl are shown in Figures 4.2 a-h. Cells grown in MCDB 202 medium gave the most linear response over all volumes for all the media used (Fig. 4.2 a-h). The affinity was consistently highest at higher volumes but the assay did not reach saturation (i.e. the level of affinity remained proportional to the solvent volume rather than reaching a plateau). Therefore, from these results it is clear that the affinity measurements may be affected by the assay method.

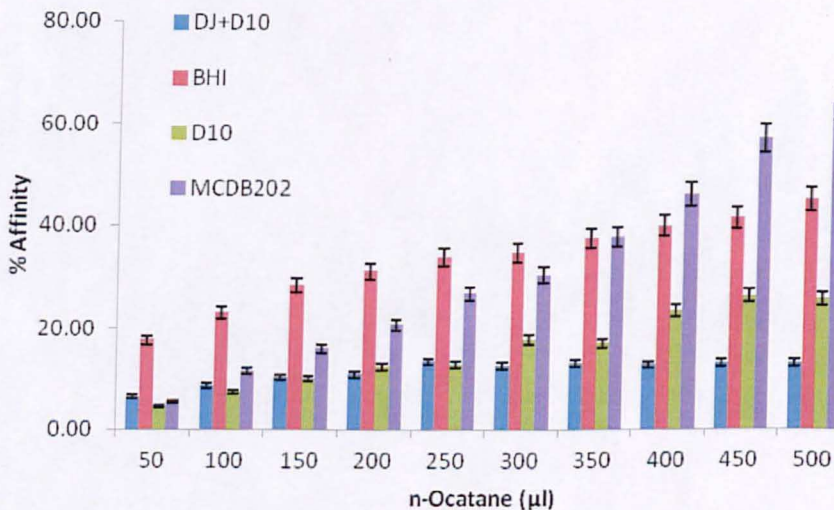
For all other media, there was some evidence of plateau for higher volumes of solvent and therefore the results gained may be more reliable as indication of potential of cells to partition into the solvent. Now cells grown in BHI did not consistently have the lowest affinity for n-octane (Fig. 4.2 a-h) as seen in Section 4.1 and overall no particular strain

Figure 4.2 Cell hydrophobicity of *L. monocytogenes* in NaCl buffer using n-octane solvent

a) Lm23074 (serotype 4b)

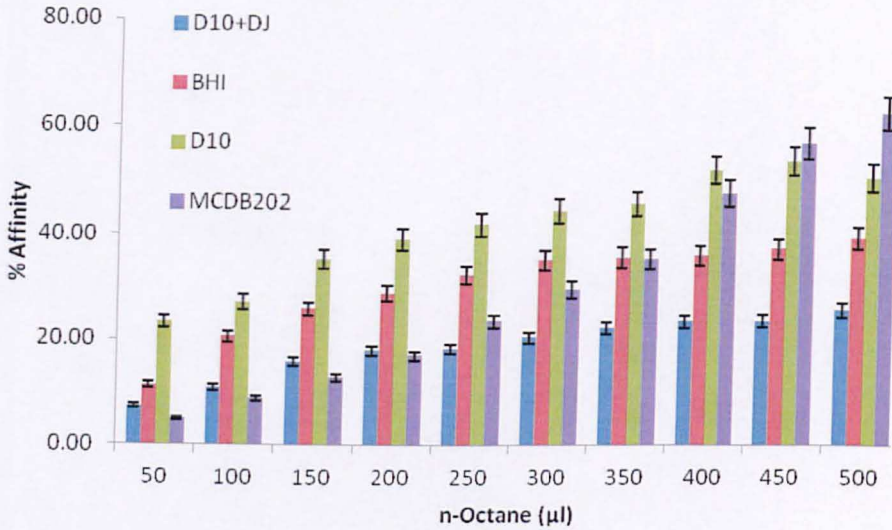


b) Lm10403S (serotype 1/2a)

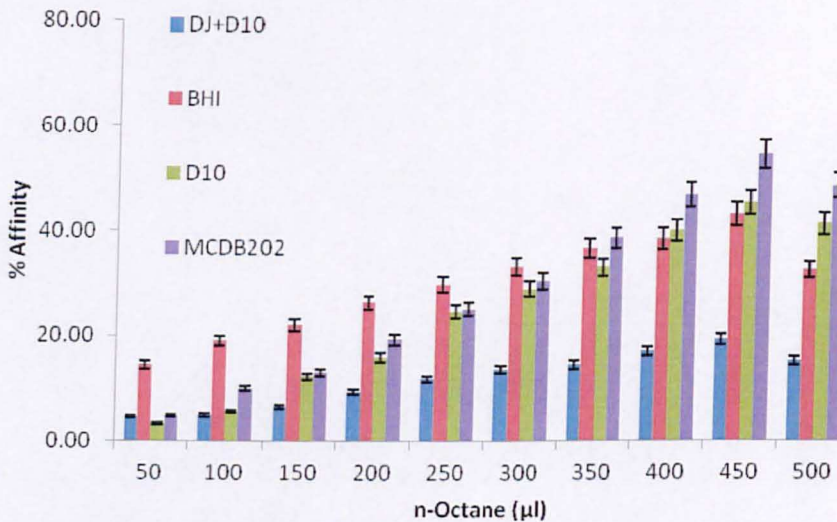


Cell hydrophobicity for cells grown in DJ+D10 (■), BHI (■), D10 (■) and MCDB 202 (■) overnight with aeration at 37 °C. Affinity to solvent n-octane was determined after vortexing for 120 s and samples being allowed to stand for 15 min. Panel (a), strain Lm 23074; Panel (b) strain Lm 10403S. Results represent values from triplicate independent samples and error bars represent 2 standard errors.

c) Lm EGD-e (serotype 1/2a)

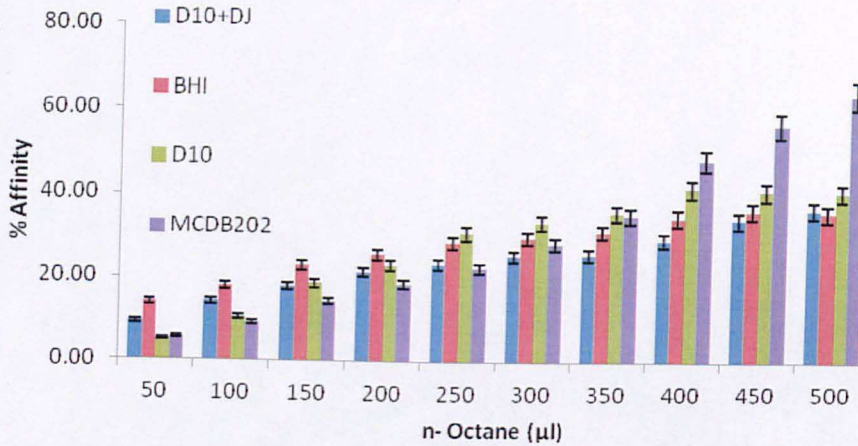


d) Lm LO28 (serotype 1/2c)

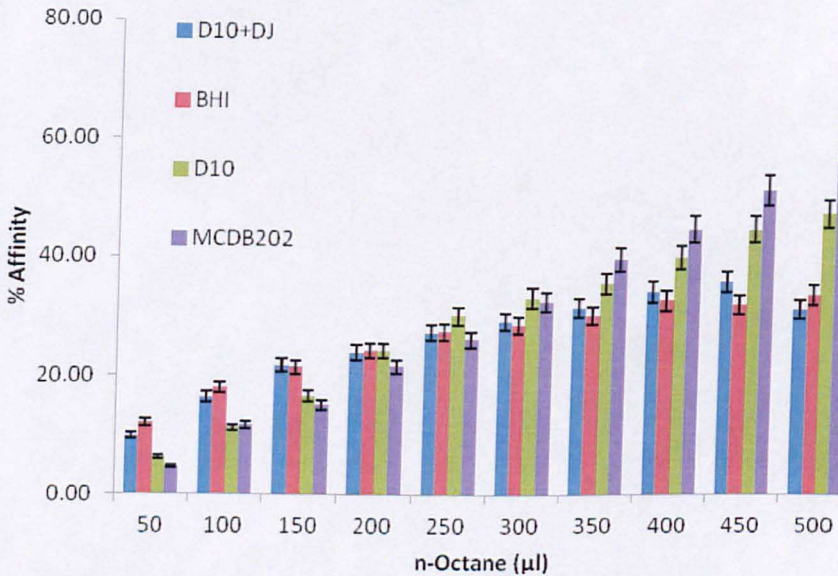


Cell hydrophobicity for cells grown in DJ+D10 (■), BHI (■), D10 (■) and MCDB 202 (■) overnight with aeration at 37 °C. Affinity to solvent n-octane was determined after vortexing for 120 s and samples being allowed to stand for 15 min. Panel (c), strain Lm EGD-e; Panel (d) strain Lm LO28. Results represent values from triplicate independent samples and error bars represent 2 standard errors.

e) Lm 00054-0305 (serotype 1/2b)

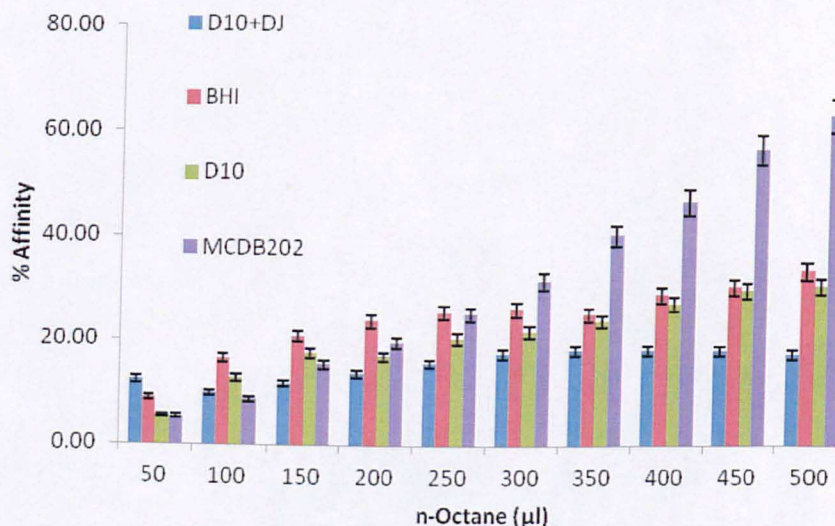


f) Lm 000622 (serotype 1/2a)

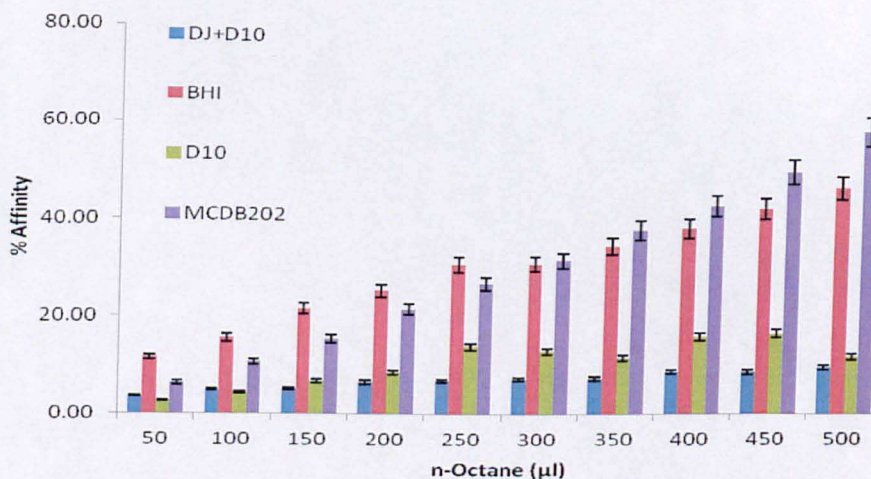


Cell hydrophobicity for cells grown in DJ+D10 (■), BHI (■), D10 (■) and MCDB 202 (■) overnight with aeration at 37 °C. Affinity to solvent n-octane was determined after vortexing for 120 s and samples being allowed to stand for 15 min. Panel (e), strain Lm 00054-0305; Panel (f) strain Lm 000622-0305. Results represent values from triplicate independent samples and error bars represent 2 standard errors.

g) Lm 00048-0305 (serotype 1/2c)



h) Lm 000101-0305 (serotype 4b)



Cell hydrophobicity for cells grown in DJ+D10 (■), BHI (■), D10 (■) and MCDB 202 (■) overnight with aeration at 37 °C. Affinity to solvent n-octane was determined after vortexing for 120 s and samples being allowed to stand for 15 min. Panel (g), strain Lm 00048-0305; Panel (h) strain Lm 000101-0305. Results represent values from triplicate independent samples and error bars represent 2 standard errors.

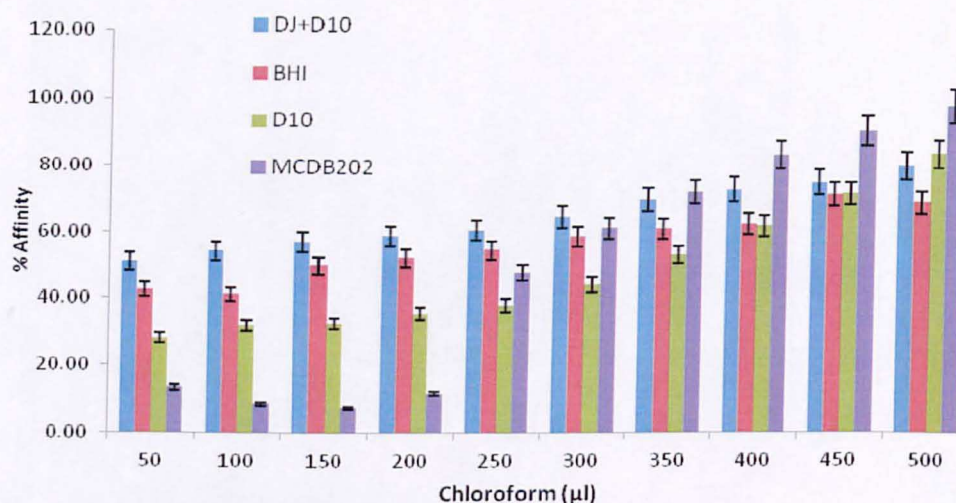
or serotype-specific pattern could be seen in the results gained showing that the strains may have different surface properties in the different media used.

The result of cell hydrophobicity of different strains carried out after growing cells in different media, overlaid with Chloroform and vortexed in 150 mM NaCl is shown in Figures 4.3 a-h. Again the most linear response was from cells grown in MCD 202 medium where the affinity was markedly different depending on the volume of the solvent used (Fig. 4.3 a-h). There appeared to be reversal of hydrophobicity at lower volumes, where at low volumes the cells appeared to be most hydrophobic and least hydrophobic at high volumes.

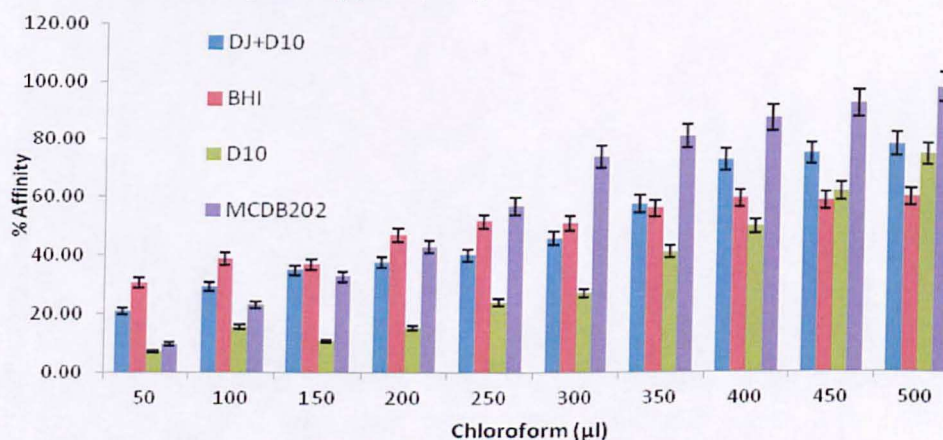
Generally the hydrophobicity of all cell types was much higher in chloroform than n-octane and three patterns emerged in the results. First, the affinity for the solvent was stable over the first 4-5 volumes (50-250 μ l) and then gradually increased (Fig. 4.3 a-h). Secondly the percentage affinity to the solvent was proportional to the volume used and finally after increasing proportional to the volume of the solvent over the 1st 4-5 volumes used (50-250 μ l), the affinity values recorded plateaued.

Figure 4.3 Cell hydrophobicity of *L. monocytogenes* in NaCl buffer using chloroform solvent

a) Lm23074 (serotype 4b)

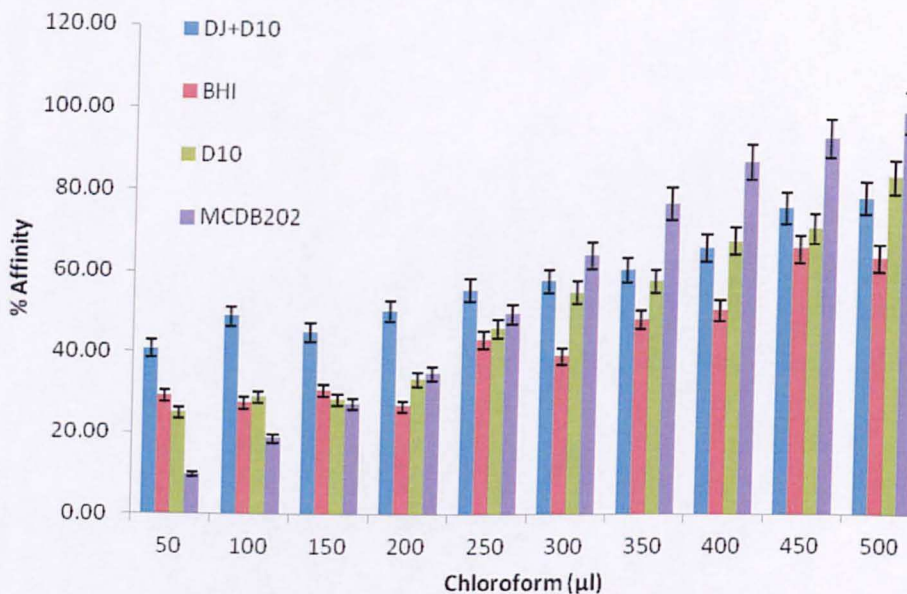


b) Lm10403S (serotype 1/2a)

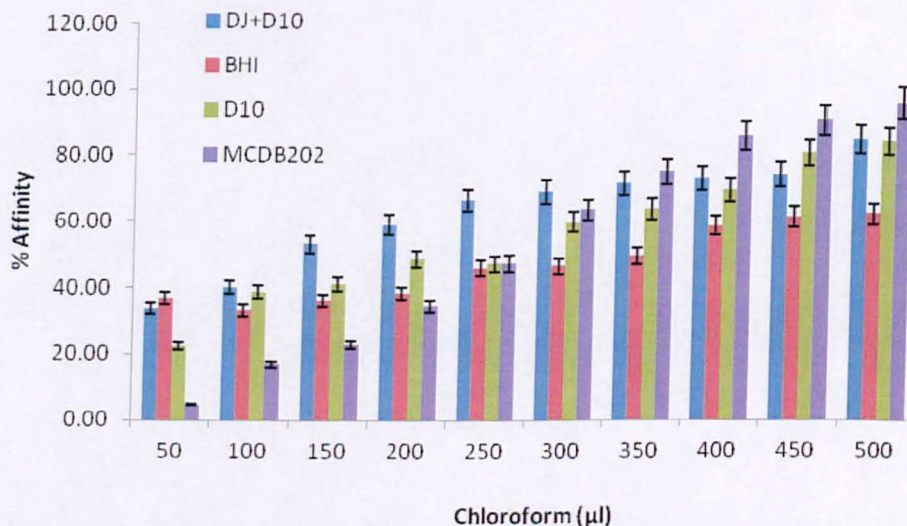


Cell hydrophobicity for cells grown in DJ+D10 (■), BHI (■), D10 (■) and MCDB 202 (■) overnight with aeration at 37 °C. Affinity to solvent chloroform was determined after vortexing for 120 s and samples being allowed to stand for 15 min. Panel (a), strain Lm 23074; Panel (b) strain Lm 10403S. Results represent values from triplicate independent samples and error bars represent 2 standard errors.

c) Lm EGD-e (serotype 1/2a)

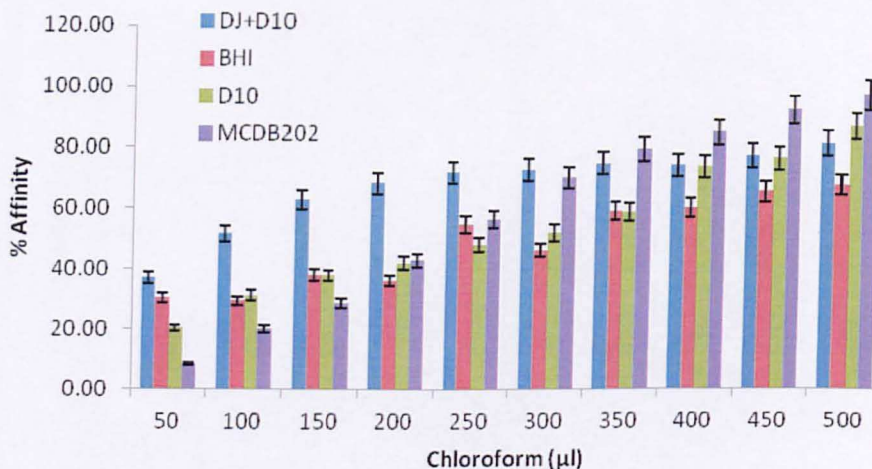


d) Lm LO28 (serotype 1/2c)

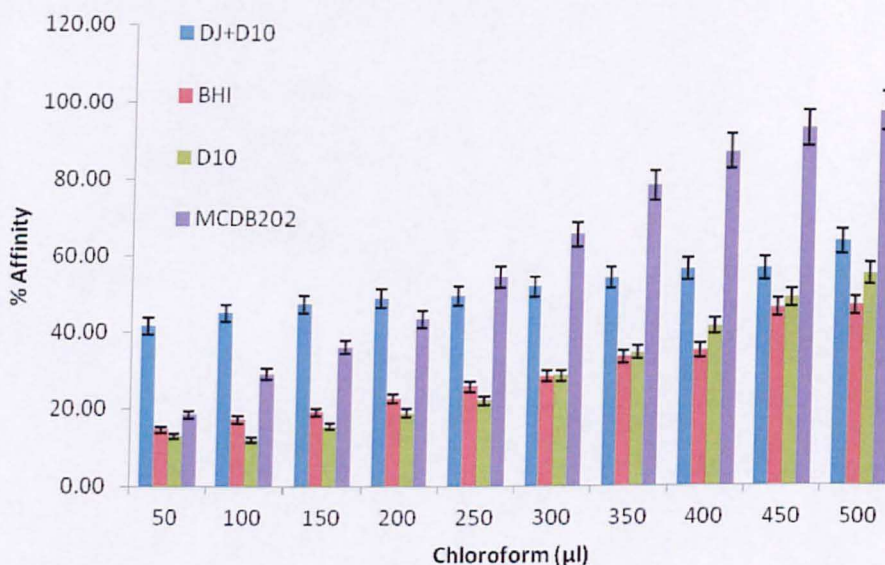


Cell hydrophobicity for cells grown in DJ+D10 (■), BHI (■), D10 (■) and MCDB 202 (■) overnight with aeration at 37 °C. Affinity to solvent chloroform was determined after vortexing for 120 s and samples being allowed to stand for 15 min. Panel (c), strain Lm EGD-e; Panel (d) strain Lm LO28. Results represent values from triplicate independent samples and error bars represent 2 standard errors.

e) Lm 00054-0305 (serotype 1/2b)

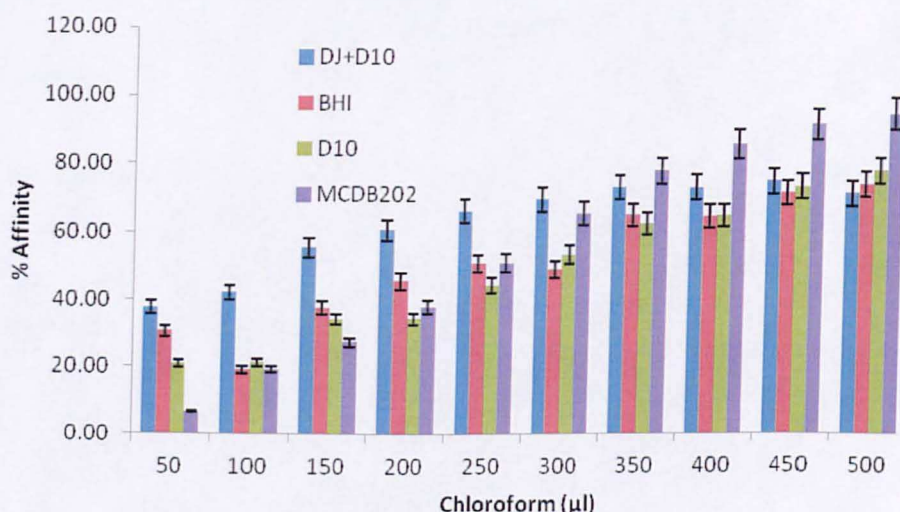


f) Lm 000622-0305 (serotype 1/2a)

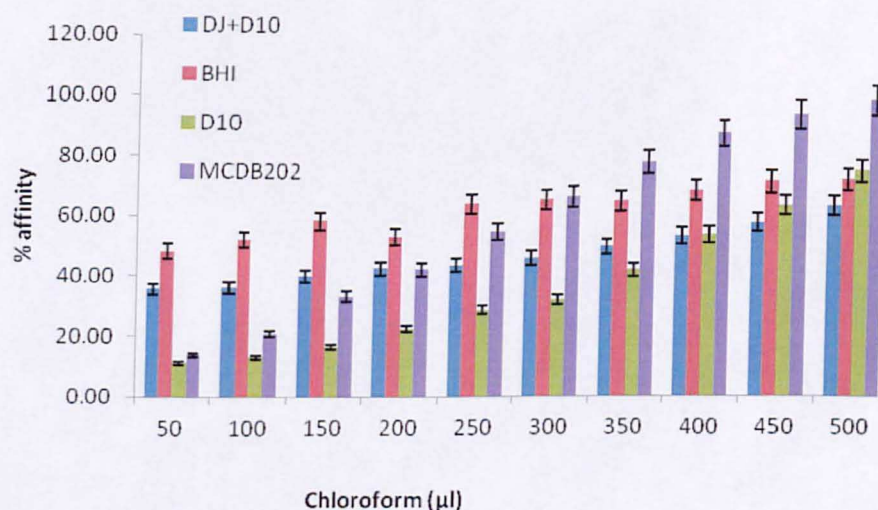


Cell hydrophobicity for cells grown in DJ+D10 (■), BHI (■), D10 (■) and MCDB 202 (■) overnight with aeration at 37 °C. Affinity to solvent chloroform was determined after vortexing for 120 s and samples being allowed to stand for 15 min. Panel (e), strain Lm 00054-0305; Panel (f) strain Lm 00048-0305. Results represent values from triplicate independent samples and error bars represent 2 standard errors.

g) Lm 00048-0305 (serotype 1/2c)



h) Lm 000101-0305 (serotype 4b)



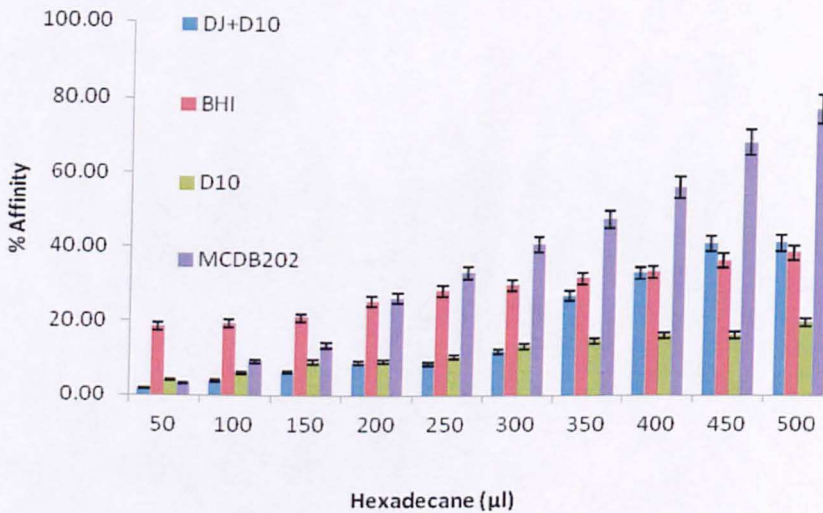
Cell hydrophobicity for cells grown in DJ+D10 (■), BHI (■), D10 (■) and MCDB 202 (■) overnight with aeration at 37 °C. Affinity to solvent chloroform was determined after vortexing for 120 s and samples being allowed to stand for 15 min. Panel (g), strain Lm 000622-0305; Panel (h) strain Lm Lm000101-0305. Results represent values from triplicate independent samples and error bars represent 2 standard errors.

Cell hydrophobicity of different strains carried out after growing cells in different media, overlaid with hexadecane and vortexed in 150 mM NaCl is shown in Figure 4.4 a-h. Affinity was generally lower in hexadecane than chloroform but higher than n-octane.

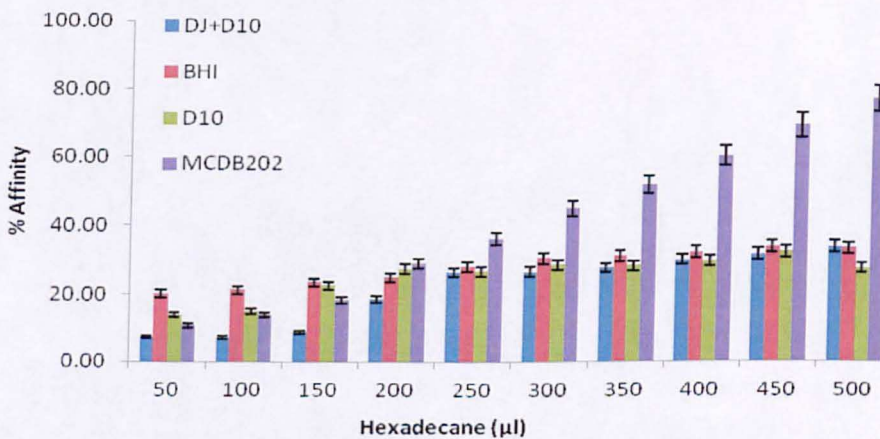
Cells grown in MCDB 202 again produced the most linear change in hydrophobicity and affinity was proportional to volume, indicating that a bigger range may be needed to reach an end point. The hydrophobicity of cells grown in other media was more stable and tended to plateau over the whole range of volumes used. In some strains affinity was lower in D10 than BHI (see Figs. 4.4a and g) but again no association with either serotype or source of the isolates was seen.

Figure 4.4 Cell Hydrophobicity of *L. monocytogenes* in NaCl buffer using hexadecane solvent

a) Lm 23074 (serotype 4b)

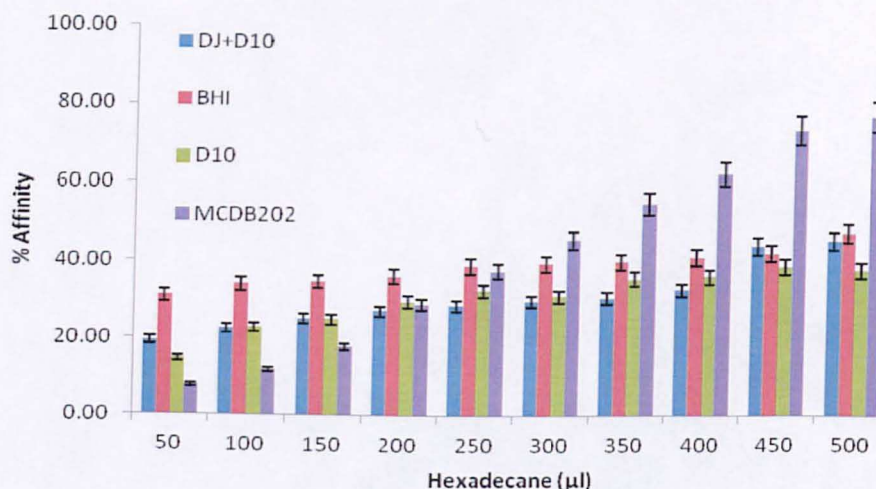


b) Lm10403S (serotype 1/2a)

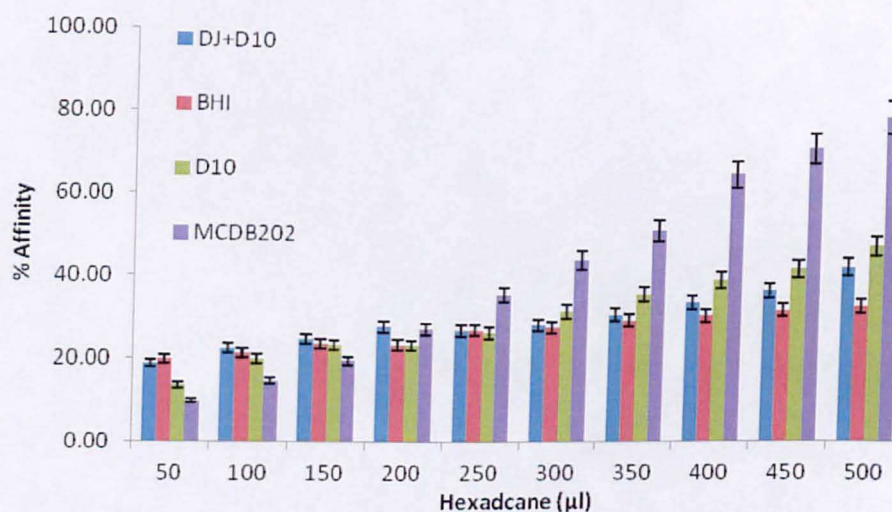


Cell hydrophobicity for cells grown in DJ+D10 (■), BHI (■), D10 (■) and MCDB 202 (■) overnight with aeration at 37 °C. Affinity to solvent hexadecane was determined after vortexing for 120 s and samples being allowed to stand for 15 min. Panel (a), strain Lm 23074; Panel (b) strain Lm 10403S. Results represent values from triplicate independent samples and error bars represent 2 standard errors.

c) Lm EDG-e (serotype 1/2a)

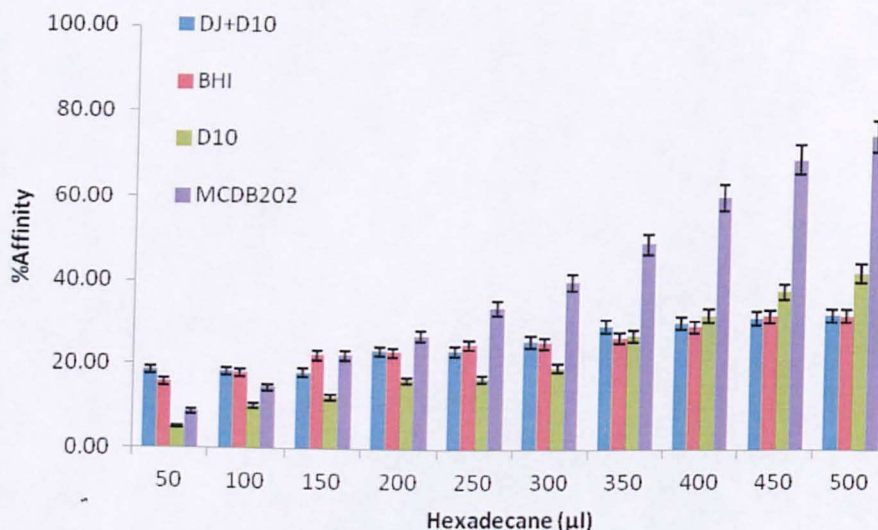


d) Lm LO28 (serotype 1/2a)

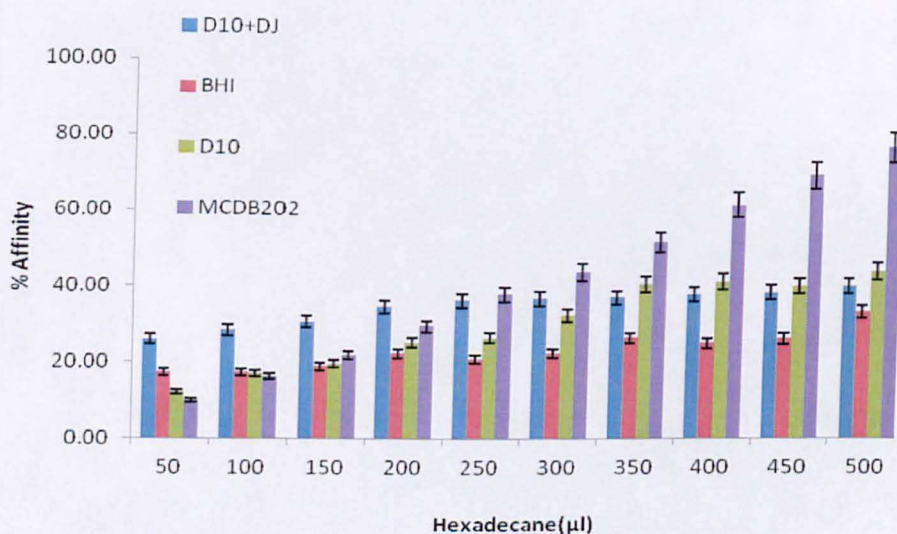


Cell hydrophobicity for cells grown in DJ+D10 (■), BHI (■), D10 (■) and MCDB 202 (■) overnight with aeration at 37 °C. Affinity to solvent hexadecane was determined after vortexing for 120 s and samples being allowed to stand for 15 min. Panel (c), strain Lm EGD-e; Panel (d) strain Lm LO28. Results represent values from triplicate independent samples and error bars represent 2 standard errors.

e) Lm 00054-0305 (serotype 1/2b)



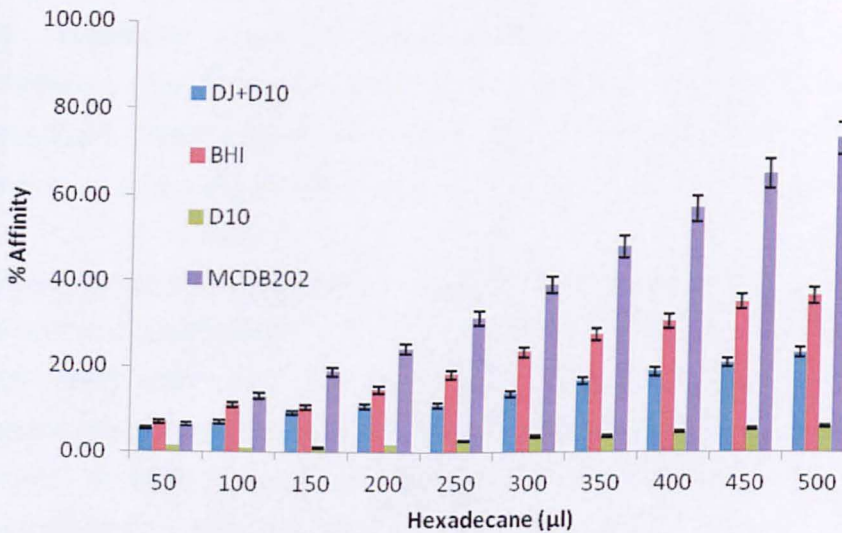
f) Lm 000622-0305 (serotype 1/2a)



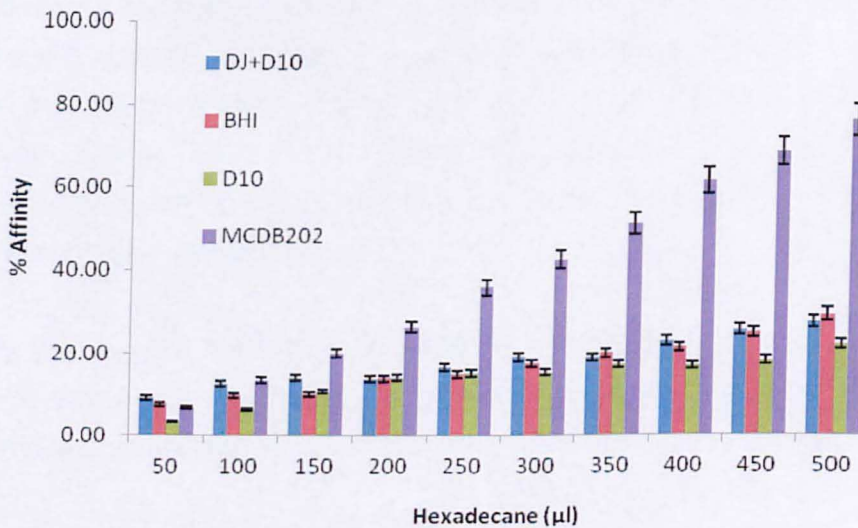
Cell hydrophobicity for cells grown in DJ+D10 (■), BHI (■), D10 (■) and MCDB 202 (■) overnight with aeration at 37 °C. Affinity to solvent hexadecane was determined after vortexing for 120 s and samples being allowed to stand for 15 min. Panel (e), strain Lm 00054-0305; Panel (f) strain Lm 000622-0305.

g) Lm 00048-0305 (serotype 1/2c). Results represent values from triplicate independent samples and error bars represent 2 standard errors.

g) Lm 00048-0305 (serotype 1/2c)



h) Lm 000101-0305 (serotype 4b)



Cell hydrophobicity for cells grown in DJ+D10 (■), BHI (■), D10 (■) and MCDB 202 (■) overnight with aeration at 37 °C. Affinity to solvent hexadecane was determined after vortexing for 120 s and samples being allowed to stand for 15 min. Panel (g), strain Lm 00048-0305; Panel (h) strain Lm 000101-0305. Results represent values from triplicate independent samples and error bars represent 2 standard errors.

The results gained showed that hydrophobicity in the volume range 50-200 μ l with NaCl buffer was at variance with using PUM buffer. This shows that the type of buffer used and its properties are a factor when assessing how the cells partition into different solvents. Considering the fact that NaCl buffer prevents charge interference (Geertsema-Doornbush *et al.* 1993), then NaCl may be the preferred buffer for performing these assays.

The hydrophobicity or affinity to solvents was highest when cells were overlaid with chloroform. This agrees with the work of Chavant *et al.* (2002) who also used 150 mM NaCl buffer when performing their experiments. They also found that chloroform, an electron acceptor solvent, always resulted in higher cellular affinity results than hexadecane or n-octane which are non-polar solvents.

The variation in hydrophobicity was not observed markedly among strains but it varied depending on the growth condition. Studies using different nutrient conditions have been shown to cause changes in the cell surface properties. In their study Briandet *et al.* (1999) used MATH assays and found that cells cultivated in TSB media supplemented with yeast extract was slightly more hydrophobic than cells cultivated in BHI.

Also Szabo *et al.* (2005) studied the factors affecting cell surface and found that in some enterotoxin producing strains of *E. coli*, their cell surfaces are modified at 30 °C and make them attach more to plastics than at 37 °C.

Geertsema-Doornbush *et al.* (1993) questioned whether the MATH assay was based on adhesion. They concluded that MATH does not measure hydrophobicity only but also measures interplay of electrostatic interactions and hydrophobicity. They found that the best affinity to hexadecane was found in the absence of electrostatic interaction or pH range close to the isoelectric point of interacting molecules. This

suggests that the electrostatic nature of the solvent used may have affected the ability of the cells to attach to the solvent.

Bellon-Fontain *et al.* (1996) proposed that MATH should be replaced with microbial attachment to solvents (MATS) since microorganisms display maximal affinity for an acidic solvent (electron donor) and low affinity for basic solvents. However, it is now generally agreed that MATH or MATS measurements are an interplay of electrostatic interactions and Van der Waals forces.

Despite the limitations of this technique for all strains, the highest recorded affinity was for cells cultivated in MCDB 202 medium. It is possible the high electrolyte content of the medium resulted in cell surfaces with the desired electrostatic nature that would encourage hydrophobic interactions with these solvents.

4.3 Effects of different vortex times and buffer volumes on cell hydrophobicity measurements.

Due to the different affinity values obtained in hydrocarbon volumes in Section 4.2., especially for the lower volume range of 50-200 μ l when using PuM and NaCl buffers and for cells grown in D10 and BHI media, an attempt was made to determine if vortex times, hydrocarbon volumes and different types of buffer affect solvent affinity.

Cultures of the strains used in Section 4.3 were prepared (see Section 3.4) and the cells resuspend in 2 ml of PuM or 150 mM NaCl buffer. Cells were overlaid with 400 μ l of chloroform, hexadecane or n-octane and then vortexed for 60 s. Cells were also overlaid with 250 μ l of the hydrocarbons and vortexed for 120 s. These conditions were chosen to represent large volume and short vortex time versus small volume and longer vortex time. Affinity to hydrocarbon was determined using the equation in Section 4.1. The experiment was limited to cells grown in BHI and D10 only in order to compare results gained between the richest and most limiting of the media used.

The results are shown in Figure 4.5 a-f. For all strains tested, the percentage partitioning into all solvents appeared to be higher when the NaCl buffer was used rather than the PuM buffer. The results suggest that the buffer used does affect how cells attach to solvents. According to Geertsema-Doornbusch *et al.* (1993) adhesion to hydrocarbon is governed by interplay of electrostatic interactions and hydrophobicity. Depending on the electrostatic interaction operating, if the attractive Van der Waals forces between the cells and water are larger than the force between the cells and the hydrocarbon, the cells will show a hydrophilic reaction. It is possible the NaCl buffer provided the desired electrostatic interaction which helped to break up the force between water and cells and made the cells more hydrophobic.

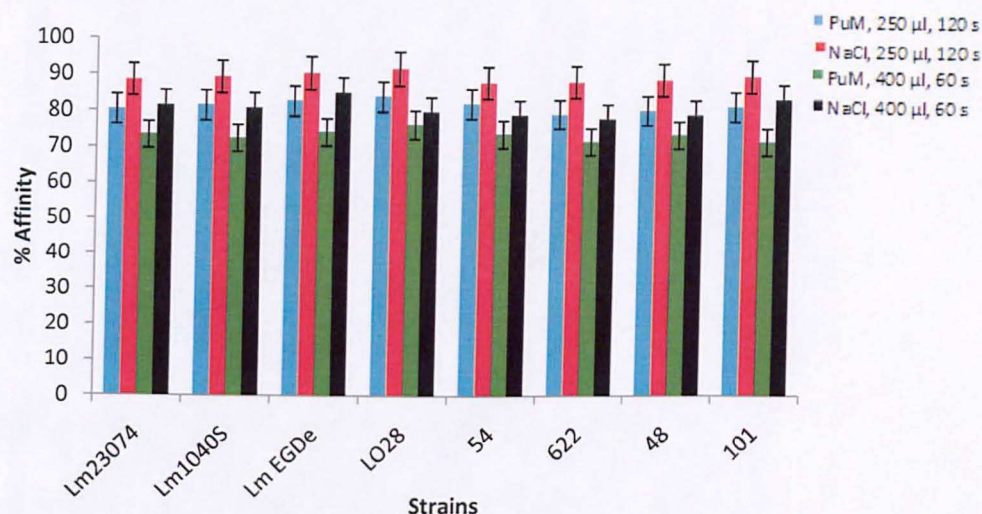
Generally the hydrophobity score was higher when the longer vortexing times were used, although the difference between the two results was not large. The only exception to this was for cells grown in BHI and vortexed with n-octane. Here a random pattern of results were seen and there was more strain variation seen in the results. However no particular pattern (i.e. correlation with serotype or laboratory strain vs environmental isolates) was evident.

When chloroform was used as solvent (Fig. 4.5 a-b), affinity for cells grown in D10 varied between 60-90% irrespective of buffer or vortex time whereas for cells grown in BHI there was gain more variation in results and the overall affinity was lower in the range of 40-60%.

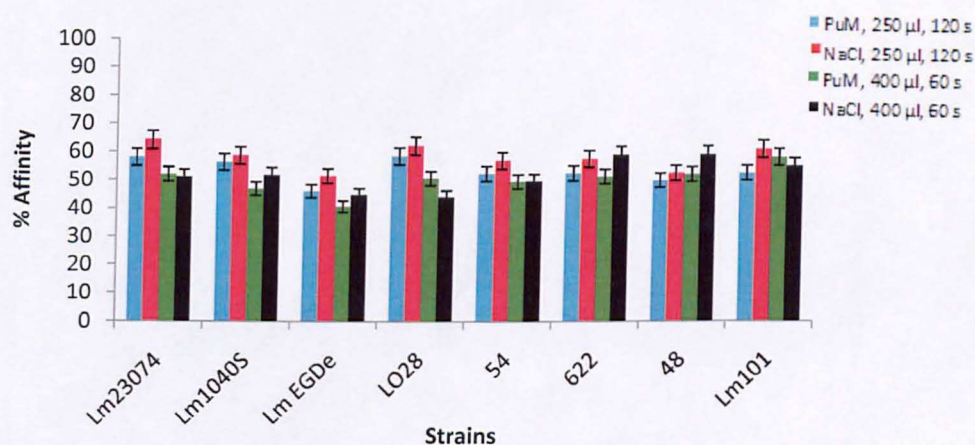
Looking at the results for hexadecane (Fig. 4.5 c-d), the same pattern was seen with cells grown in D10 scoring having percentage affinity between 50-80% and those in BHI between 25-40%. Interestingly again more strain

Figure 4.5 Hydrophobicity assays using different vortex times, volumes and buffers.

a) Cells grown in D10 mixed with chloroform

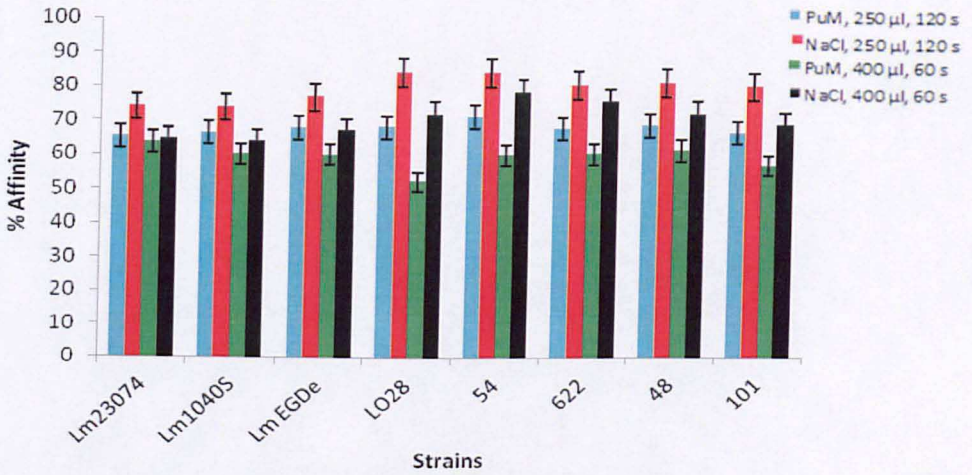


b) Cells grown in BHI mixed with chloroform

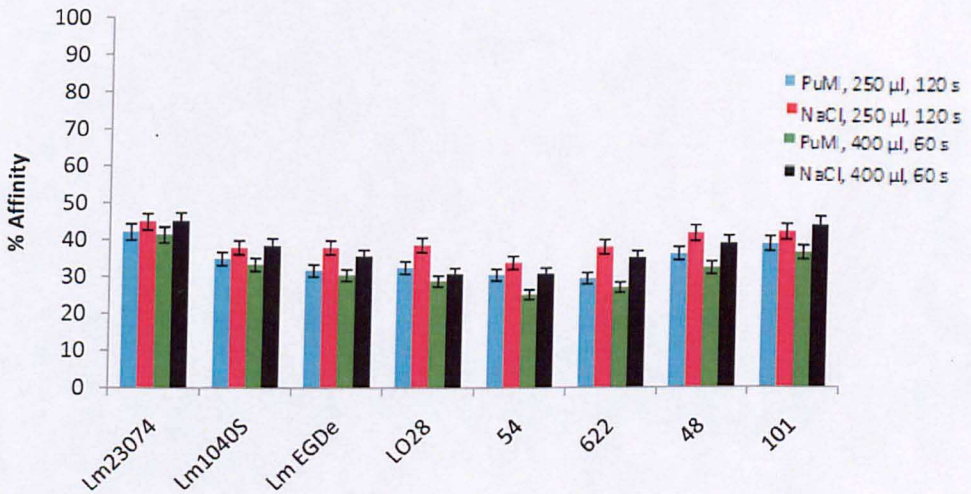


Cells were grown in either D10 (a) and BHI (b) vortexed with chloroform. Variables used were resuspended in either PuM (■, ■) or NaCl buffers (■, ■), either 250 µl (■, ■) with 120s vortex time or 400 µl (■, ■) of solvent and 60 s vortex time. Results represent values from triplicate independent samples and error bars represent 2 standard errors.

c) Cells grown in D10 mixed with hexadecane

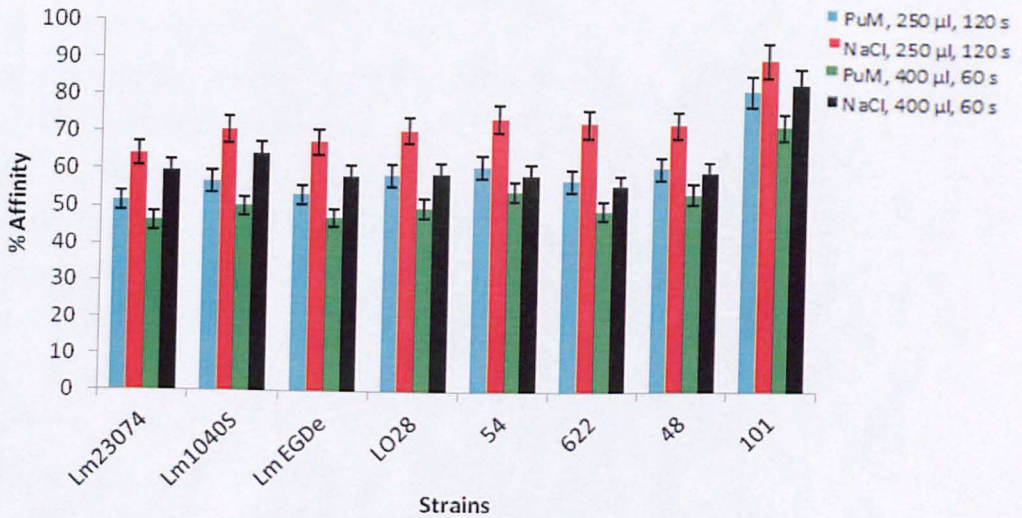


d) Cells grown in BHI mixed with hexadecane

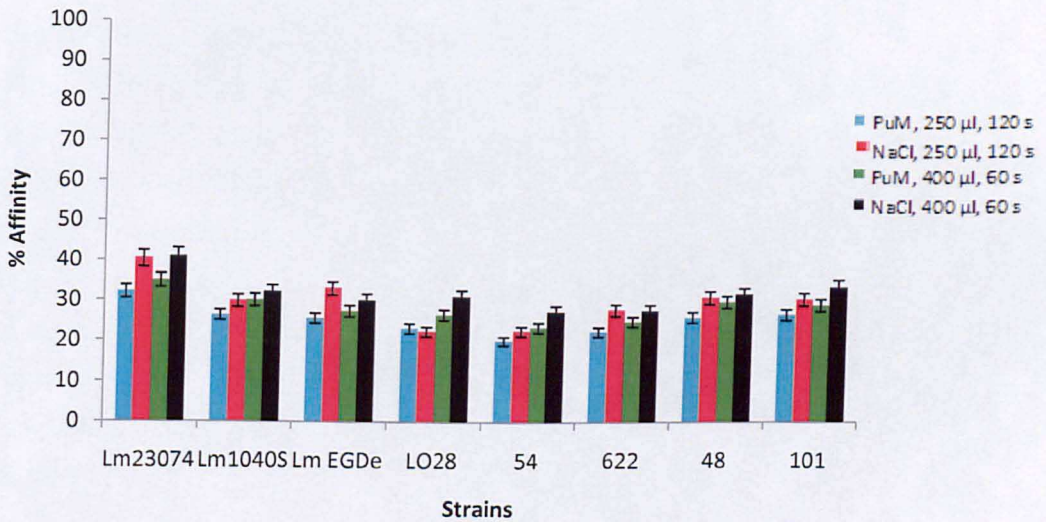


Cells were grown in either D10 (c) and BHI (d) vortexed with hexadecane. Variables used were resuspended in either PuM (■, ■) or NaCl buffers (■, ■), either 250 µl (■, ■) with 120s vortex time or 400 µl (■, ■) of solvent and 60 s vortex time. Results represent values from triplicate independent samples and error bars represent 2 standard errors.

e) Cells grown in D10 mixed with n-octane



f) Cells grown in BHI mixed with n-octane



Cells were grown in either D10 (e) and BHI (f) vortexed with n-octane. Variables used were resuspended in either PuM (■, ■) or NaCl buffers (■, ■), either 250 µl (■, ■) with 120s vortex time or 400 µl (■, ■) of solvent and 60 s vortex time. Results represent values from triplicate independent samples and error bars represent 2 standard errors.

variation was evident for cells grown in BHI with no clear pattern of the basis of this variation with strain type.

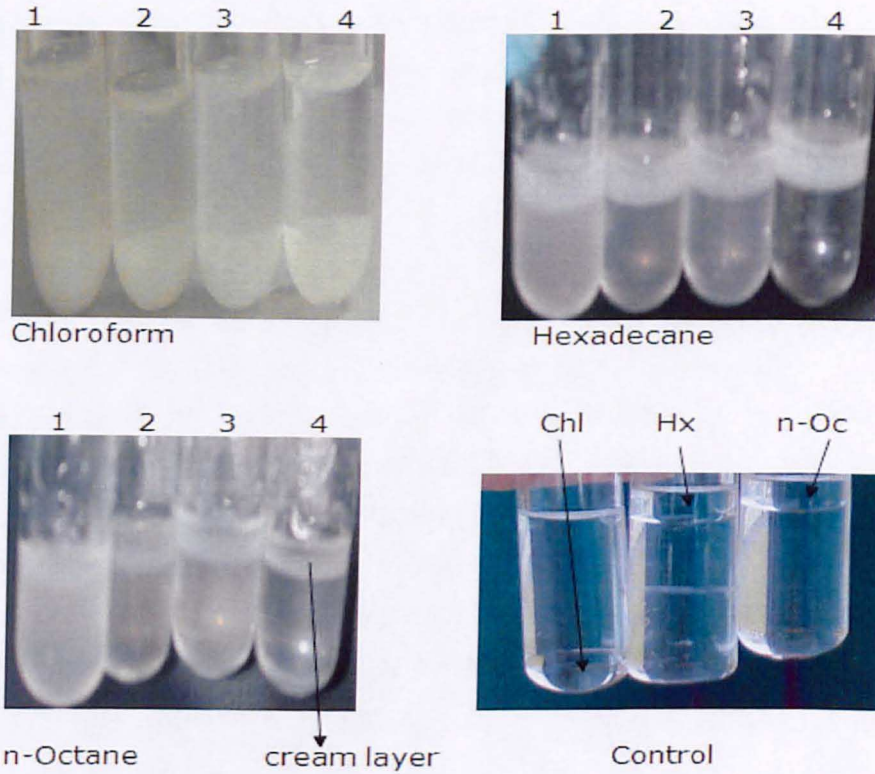
For cells vortexed with n-octane the same pattern of higher hydrophobicity scores for cells grown in D10 (40-70% range) than those grown in BHI (20-40% range) was seen. Given that it was clear that the MATH assay results are very sensitive to volume variation, buffer and vortex time, cells grown in D10 consistently had higher affinity than cells grown in BHI when PuM buffer was used but varied among species when NaCl buffer was used. For some species, affinity was higher for cells grown in BHI than D10 especially in lower volumes. Longer vortex times may affect affinity since it was found that overall and irrespective of media or buffer used, cells suspended in NaCl buffer and vortexed for 120 s had higher affinity 75% of the time.

4.4 Effect of cells on separation of phases

Since there seemed to be effects of the cell growth media on the partitioning of the cells into aqueous and non-aqueous layers after vortexing, the efficiency of phase separation was investigated for the three hydrocarbons. The results of the vortexing assays and a control experiment where no cells were added to the aqueous buffer are shown in Figure 4.6. In the absence of any cells, the hydrocarbon formed a discrete layer in the tube. For hexadecane and n-octane the non-aqueous layer formed the upper layer while chloroform formed the bottom layer.

However in the presence of the cells, the non-aqueous and aqueous layers were less demarcated and stable cream layer was formed at the interface of the two layers made up of cells and a hydrocarbon/water emulsion.

Figure 4.6 Images of MATS assays



Images of cell hydrophobicity assays of cells grown in BHI (1) D10 (2) D10+DJ (3) and MCDB 202 (4) suspended in 2 ml of 150 mM NaCl and mixed with 500 μ l of Chloroform (Chl), Hexadecane (Hx), or n-Octane (n-Oc) solvents. After vortexing formation of a stable cream emulsion layer was observed. As a control, solvents were mixed with 150 mM NaCl without *Listeria* cells. Chloroform settled at the bottom of the tube while Hexadecane and n-Octane formed an upper layer, and the aqueous and non-aqueous layers are clearly demarcated.

4.5 Phase contrast microscopy of samples from MATH assays

From the results presented in Section 4.3, it was clear that the surface properties of the bacteria were affecting the amount of partitioning and the formation of stable emulsions was evident. To find out if the cells were staying on the surface of the oil-water emulsion or actually partition into the oil droplets within the emulsion, phase contrast microscopy was used.

Cells of strains Lm 10403S were prepared as in Section 3.4. Harvested cells were grown overnight in BHI, MCDB 202, D10 and DJ+D10 at 37 °C with shaking. The cells grown in each media were resuspended in 2 ml of 150 mM NaCl buffer after which the cells were overlaid with 500 µl of hexadecane, n-octane or chloroform and then vortexed for 120 s. After vortexing, the samples were left standing for 15 min to allow them to separate into aqueous and non aqueous layers. As shown in Figure 4.6, a stable cream layer was formed with no clear layer of solvent visible.

Wet mounts of cells from each layer or phase were prepared by placing a drop on a clean glass slide then covered with a cover slip. These were viewed using oil Immersion objective (Phase ring 100 and 1000 x magnification).

Results of phase contrast microscopy for samples taken for cells grown in BHI, MCDB 202, D10 and DJ+D10 and vortexed in hexadecane are shown in Figure 4.7. Generally, the distribution of cells in the aqueous phase was uniform and the same pattern was observed for samples taken from the aqueous layer (upper panel Figs. 4.7-4.9).

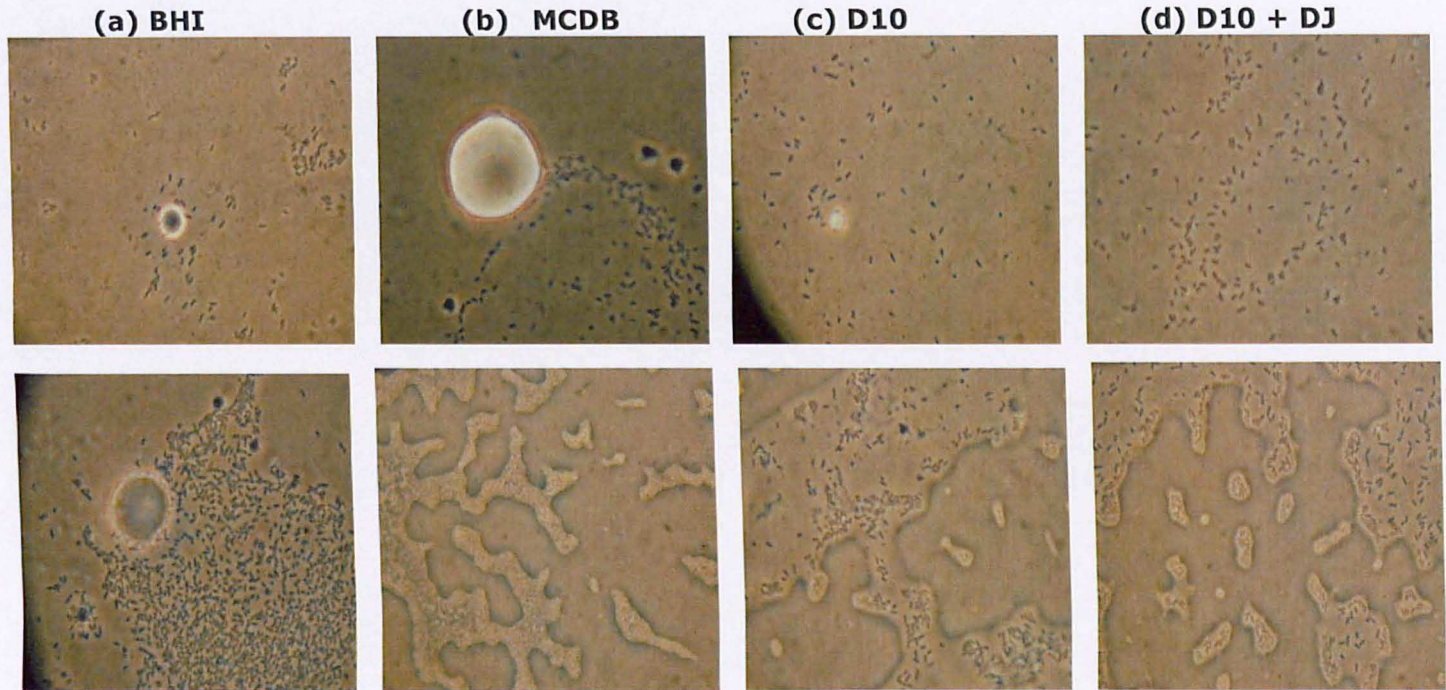
The samples from the non-aqueous phase vortexed in chloroform (Fig. 4.9) appeared to contain the highest density of cells. This matches the results gained in the MATHS assays where most partitioning was seen in chloroform (See Figs. 4.2 and 4.5). What was clear from these observations was that the bacteria were not uniformly distributed in

the solvent, but from these results it was not clear if the cells were aggregating in the aqueous or non aqueous part of the emulsion.

The BHI cell distribution in non-aqueous phase appears different to rest of the results. Cell aggregation was lower when compared to other media and this may explain the low affinity results experienced in Section 4.2.

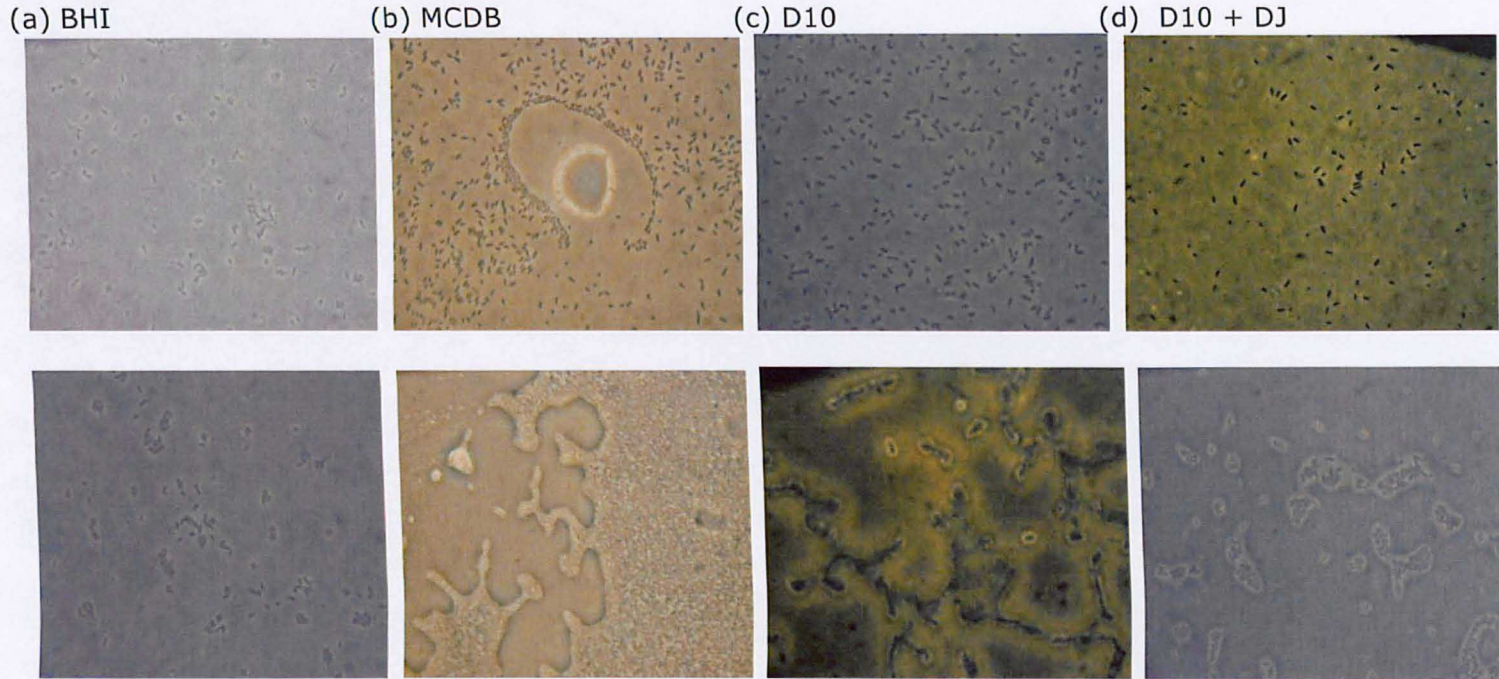
It was then necessary to view the cell distribution in a more three dimensional way and so confocal scanning laser microscopy was used to determine how the cells were interacting with the water/solvent emulsion.

Figure 4.7 Cells from aqueous and non-aqueous layer vortexed with hexadecane



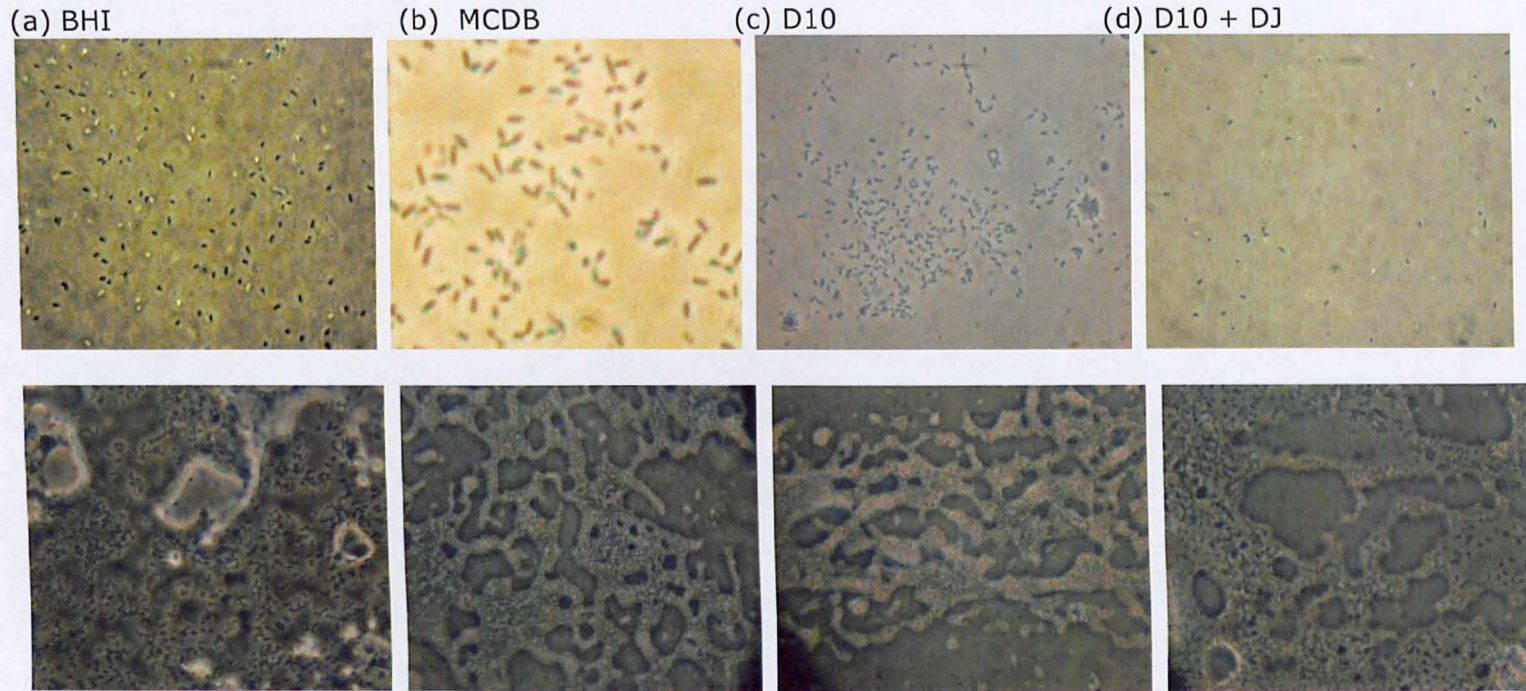
Cells of *Listeria* from aqueous (top) and corresponding non aqueous (below) layers of phase separated cells grown in BHI (a), MCDB202 (b), D10 (c) and DJ+D10 (d). The cells were loosely distributed and few in the aqueous phase but was packed in the non-aqueous phase.

Figure 4.8 Cells from aqueous and non-aqueous layer vortexed with n-octane



Cells of *Listeria* from aqueous (top) and corresponding non aqueous (below) layers of phase separated cells grown in BHI (a), MCDB202 (b), D10 (c) and DJ+D10 (d). The cells were loosely distributed and few in the aqueous phase but was packed in the non-aqueous phase. Clumping was observed for cells grown in D10 in the solvent phase (lower panel c)

Figure 4.9 Cells from aqueous and non-aqueous layer vortexed with chloroform



Cells of *Listeria* from aqueous (top) and corresponding non aqueous (below) layers of phase separated cells grown in BHI (a), MCDB202 (b), D10 (c) and DJ+D10 (d). The cells were loosely distributed and few in the aqueous phase but was packed together in the non-aqueous phase.

4.6 Confocal Microscopy of *Listeria* cells grown in different media and vortexed with Chloroform

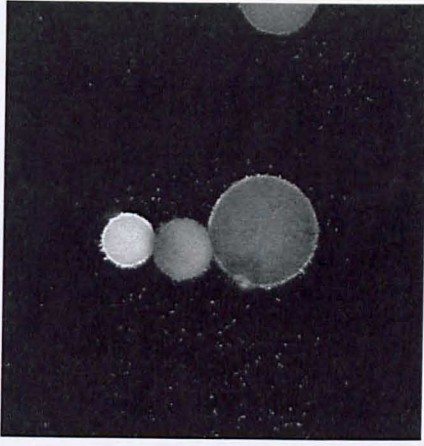
According to Hass *et al.* (2001) confocal microscopy offers several advantages over other conventional microscopic techniques as a tool for studying the interaction of bacteria and food surfaces. Studies can be carried out without sectioning the specimen and microbial attachment and detachment to surfaces can be made *in situ*. Furthermore, it allows 3 dimensional images of the cells or biofilm thus providing more information for analysis (Carlson *et al.* 1989; Vershure *et al.* 1997). The main aim of the confocal microscopic analysis performed here were to determine how the cells were distributed in the emulsion.

Cells of strains Lm 10403S were used for the confocal microscopy to maintain consistency. Cells were grown in BHI for 18 h and the transferred into BHI, D10, DJ+D10 and MCDB 202 (see Section 3.4). The cells were then resuspended in 2 ml 150 mM NaCl and overlaid with 500 µl of chloroform before vortexing for 120 s. The cells were allowed to stand for 15 min before slides were prepared from the non-aqueous phase using rhodamine as diluent and viewed using a confocal laser microscope at various magnifications (section 2.13).

The results are shown in Figures 4.10-4.12. The attachment pattern was the same for all cells grown in different media (BHI, D10, D10+DJ, MCDB 202) but the degree of attachment varied. It was observed that the cells were interacting with the surface of the solvent and were not inside the solvent droplets but formed layers surrounding them. At higher magnification the shapes of the cells were clearly defined showing that the bright fluorescent layer seen at lower magnifications was due to bacteria cells (Figs. 4.11 and 4.12). Cells grown in the MCDB 202 medium (Fig. 4.10) seems to form a brighter layer than the cells grown in the other media.

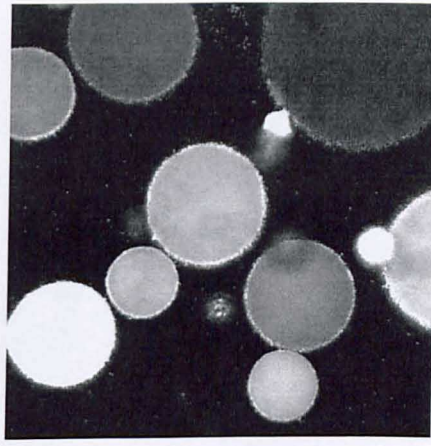
Figure 4.10 Confocal microscopy of non-aqueous phase

(a)



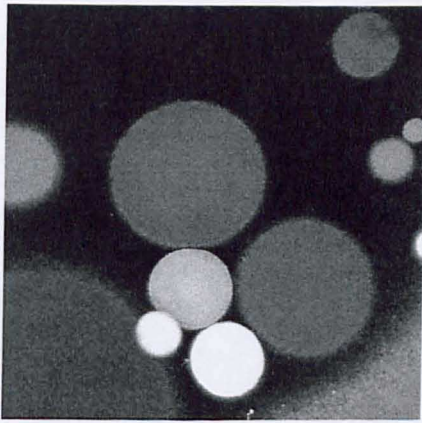
1.2 mm

(b)



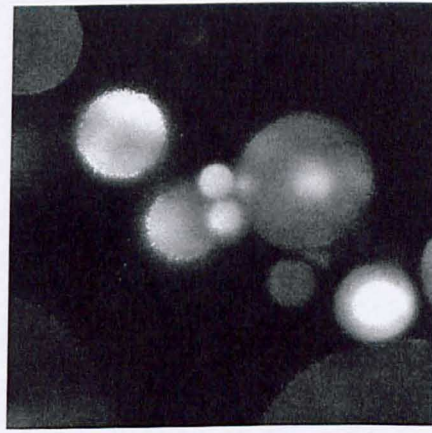
1.2 mm

(c)



1.2 mm

(d)

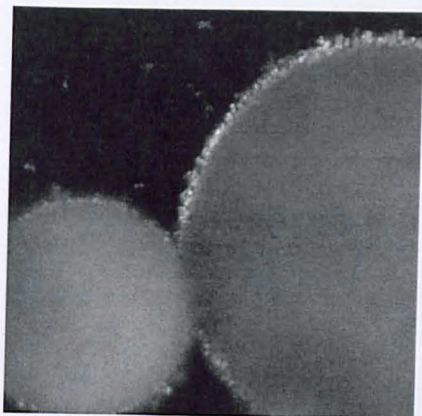


1.2 mm

Confocal microscopy analysis at 1.2 mm for cells grown in BHI (a), MCDB 202 (b), D10 (c) and DJ+D10 (d) and vortexed in chloroform. Confocal laser microscopy images were collected using a Nikon Eclipse Ti inverted Confocal microscope (Nikon UK Ltd) and EZ-C1 control software was used for analysis of data collected.

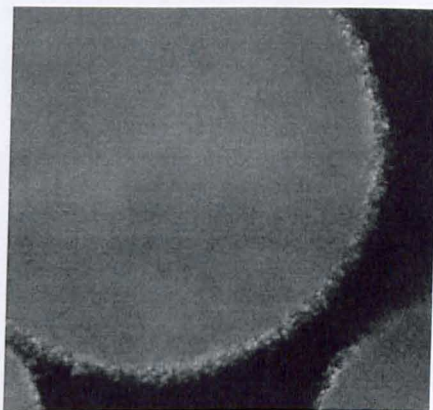
Figure 4.11 Confocal microscopy with increased optical sectioning

(a)



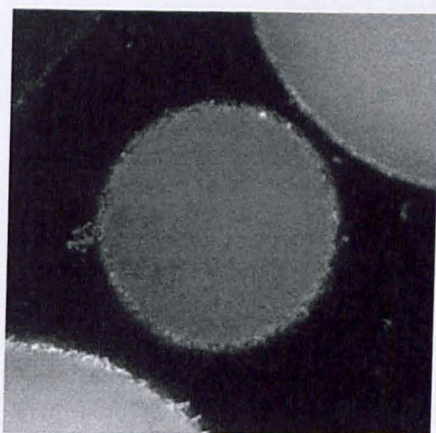
300 μm

(b)



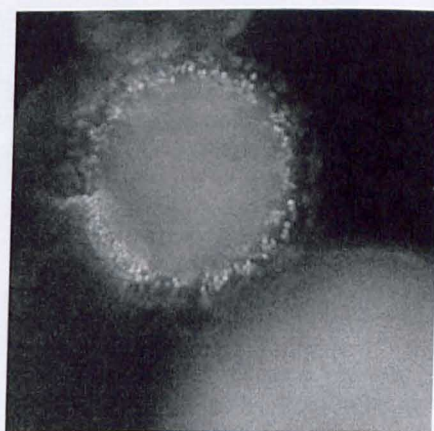
300 μm

(c)



300 μm

(d)



300 μm

Confocal microscopy analysis at 300 μm for cells grown in BHI (a), MCDB 202 (b), D10 (c) and DJ+D10 (d) and vortexed in chloroform. Confocal laser microscopy images were collected using a Nikon Eclipse Ti inverted Confocal microscope (Nikon UK Ltd) and EZ-C1 control software was used for analysis of data collected.

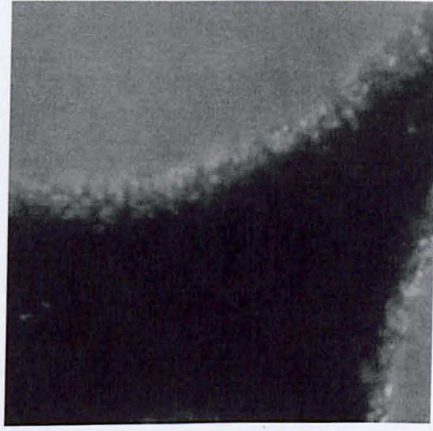
Figure 4.12 Confocal microscopy of cells on oil droplets

(a)



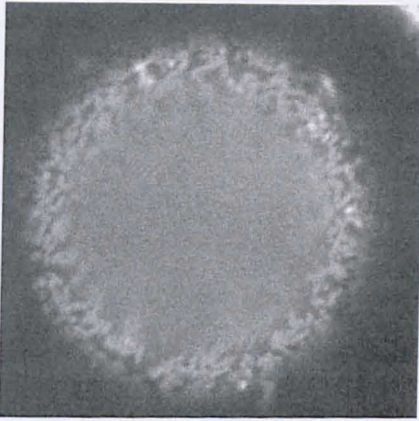
150 μ m

(b)



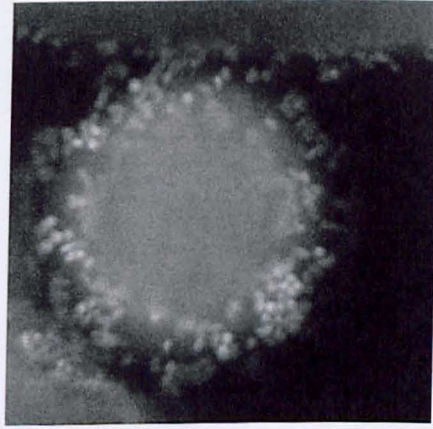
150 μ m

(c)



150 μ m

(d)



150 μ m

Confocal microscopy analysis at 150 μ m for cells grown in BHI (a), MCDB 202 (b), D10 (c) and DJ+D10 (d) and vortexed in chloroform. Confocal laser microscopy images were collected using a Nikon Eclipse Ti inverted Confocal microscope (Nikon UK Ltd) and EZ-C1 control software was used for analysis of data collected.

4.7 Conclusions

Results show that bacteria are interacting with the droplet surface and stabilizing the emulsion. It explains why MATH assays produced cream layer and also why results may vary with method and solvent. It suggests surface of *Listeria* was behaving as an emulsifier. Properties of these are often amphoteric (i.e with one part charged and one part hydrophobic). Fluorescence of cells grown in MCDB 202 was apparently more intense and would agree with the MATH assay results where the generally the highest affinities to the solvent were recorded.

The fact cells grown in different media were behaving differently agreed with the idea that surface was being modified but at this stage, no clear property could be seen to account for these changes.

Chapter 5 RESULTS:

Physical Evidence of an extra cellular polymeric substance (EPS)

5.1 Introduction

In the Hydrophobicity assay (Chapter 4) the cells grown in minimal media were seen to partition differently into solvents and to be able to stabilise emulsions. One explanation for this is that the cells were changing their surface properties through the production of EPS. Hence the main aim of the work described in this Chapter was to establish whether the presence of any EPS capsular material could be detected that could account for these changes.

A number of microscopic techniques used in this study have been used extensively to study bacterial cell shape and morphology. These include bright-field light microscopy, phase contrast microscopy, and scanning electron microscopy. For the bright field microscopy, the cells were first stained with Nigrosin or Giemsa, both of which have been used previously to detect the presence of capsular material.

5.2. Nigrosin capsule stain

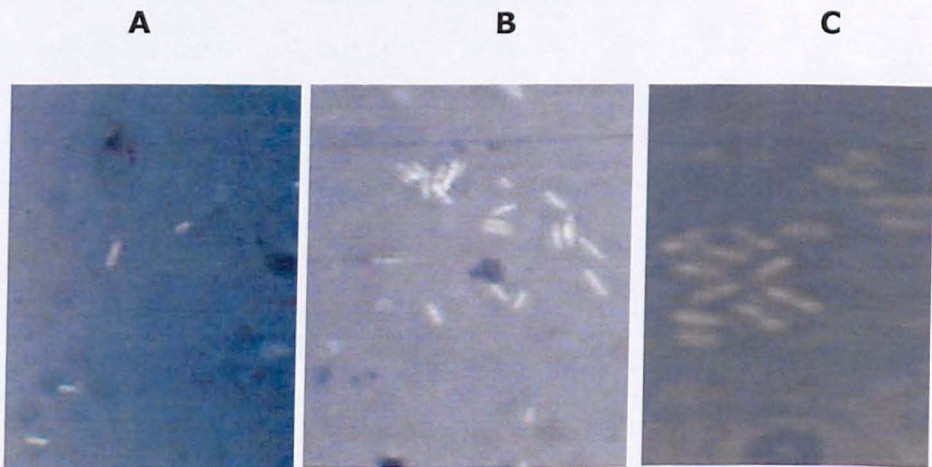
Nigrosin staining was carried out as described by Kapale *et al.* (2003). These authors pointed out that the standard Indian ink stain capsule stain for bacteria described by Duguid (1989) is not very satisfactory since the cell bodies of the bacteria are not stained with the Indian ink which makes them less visible using ordinary light microscopy. Instead they recommended the use of the Nigrosin stain which is a modification of the Indian ink stain.

Cells of strain Lm 10403S were prepared as described in Section 3.4 to create high cell density inocula for the different growth media to be used. The washed cells were then grown for 24 h in BHI, D10 or MCDB 202. The other medium (D10+DJ) was excluded to avoid materials from the duck juice preparation that could interfere with the staining. Nigrosin staining was performed by placing a thin film of bacteria on a

microscope slide and then heat fixing the sample before covering with 1:3 carbol fushin (Section 2.14). The carbol fushin was washed off and then the Nigrosin stain applied without washing off and allowed to dry before the samples were viewed using a x100 immersion oil objective.

The results (Fig. 5.1) showed that capsular material was evident surrounding cells grown in all the media for 24 h. After Nigrosin staining the rod-shaped *Listeria* cells were seen to be surrounded by clear zones indicating the presence of capsular material. The clear zones were similar in appearance to the capsule stains of rod shaped *E. coli* K29 observed by Koval and Bayer (1997) when they used Indian ink to stain cells of *E. coli* K29 grown in Luria broth.

Figure 5.1 Nigrosin stains of cells grown for 24 h



Nigrosin stain of Lm 23074 cells grown overnight with shaking in BHI (A), D10 (B) and MCDB 202 (C) at 37 °C. Staining was achieved by heat fixing cells, and then stained with carbol fuschin before staining with Nigrosin dye and imaged using a x100 immersion oil objective. Cells with capsules (negative stain) appear shiny against a dark back ground.

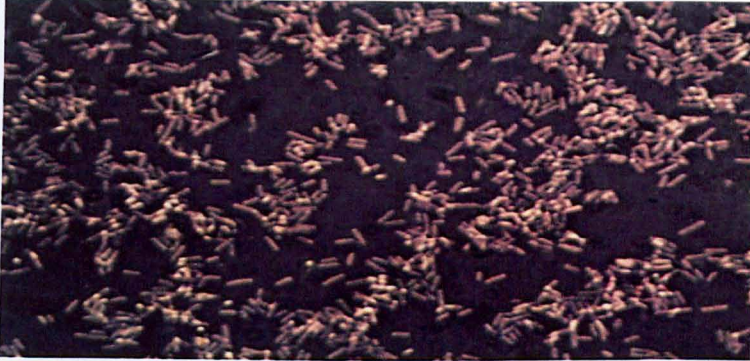
5.2.1 Nigrosin capsule stain after 10 days

It has been reported that cells form more EPS, which favours biofilm formation, as cultures grow older and become nutrient limited. For instance it has been shown by Di Bonaventura (2008) that production of biofilms and EPS is influenced by environmental growth conditions. To try and enhance the amount of EPS produced by these broth grown cells they were left for another 10 days in the richest media (BHI; defined by that producing the fastest growth rate) and also in the most minimal media D10 as a comparison.

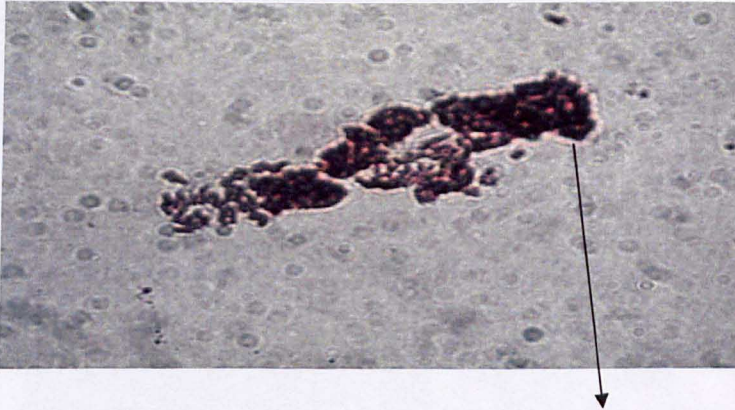
After extended incubation of these cultures, the opaque capsule layer surrounding the cells grown in D10 was very pronounced and there was more evidence of cell clumping in these cultures (Fig. 5.2b). This was less evident for cells grown in BHI (Fig. 5.2a) but the presence of some areas of brightness where cells clumped together suggested that some EPS was being made in these aged cultures. Interestingly Chavant *et al.* (2002) suggested that biofilms form best when cells are not grown in rich BHI medium. The results obtained here are consistent with that observation if this capsular material aids attachment and development of biofilm.

Figure 5.2 Nigrosin stains of cells of strain after 10 d incubation

(a)



(b)



Shiny capsule material surrounding clumped cells

Light microscopy (x100 immersion oil objective) images of Nigrosin stains of strain Lm 10403S grown for 10 days in BHI (panel a) and D10 media (panel b). Cells were grown at 37 °C with shaking.

5.2.2 Formation of *Listeria* EPS in fresh cultures

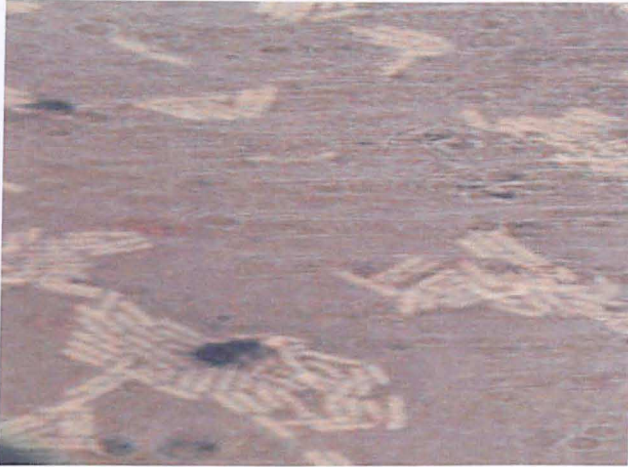
Due to the observation of EPS material developing on cultures grown in BHI for extended periods of time an attempt was made to get rid of the old population of cells in the broth culture and to monitor EPS formation in fresh cultures. It was hoped that by doing this the difference in EPS levels between the different samples would be clearer.

To generate populations of cells, Lm 10403S was prepared as in Section 3.4 and inoculated into BHI broth to an initial OD_{600nm} of 0.05 and then grown to OD_{600nm} 0.5 (the strain was chosen because it is widely used reference strain in literature). This was then diluted again to an OD_{600nm} of 0.05 and then regrown to OD_{600nm} of 0.5 (cycled cells). Nigrosin stains were prepared from samples taken after the first and second cycle at OD_{600nm} of 0.5.

Results (Fig. 5.3) show *Listeria* cells covered with EPS capsular material was formed in both cycles indicating that the capsule production is not just synthesised in older, nutrient starved cultures.

Figure 5.3 Nigrosin stains from two growth cycles

A



B



Cells of Lm 10403S grown in BHI from 0.05 OD_{600nm} to 0.5 OD_{600nm} (panel A). The cells were diluted again to 0.05 OD_{600nm} and grown to 0.5 OD_{600nm} (panel B). The capsules are evident on cells taken from both cycles. Cells with capsules (negative stain) appear shiny against a dark back ground.

5.2.3 Initial formation of capsular material

To determine the time of onset of capsule formation following transfer into the new media, the cells were grown twice to OD_{600nm} 0.5 to generate a population of cells with the majority of the cells in the exponential phase of growth, then harvested by centrifuging at 3000 x g and placed in 10 ml of D10, BHI and MCDB 202 broth and were then grown at 37 °C with shaking. Samples were taken from these after 0, 20, 40, and 60 min intervals and then after overnight (18 h) incubation. The cells were heat fixed on a microscope slide and stained with Nigrosin stain as described in Section 5.2.

The results are shown in Figures 5.4-5.6. There was some evidence of increased capsule formation in D10 but cells grown in BHI also had some capsule formation. It was concluded that the staining method was not sufficiently sensitive to quantify differences in capsule synthesis on individual cells.

Figure 5.4 Nigrosin stain immediately after cell cycling

a) BHI

b) MCDB202



c) D10

d) D10

Listeria rods and capsular EPS

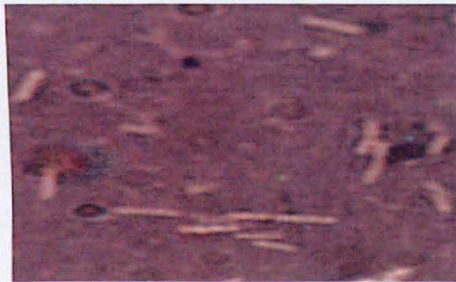
Cells of Lm 10403S after cycling were introduced into different types of fresh media; BHI (panel a), MCDB 202 (panel b) or D10 (panels c and d) and then stained with Nigrosin before imaging using a x100

Figure 5.5 Nigrosin stains after 20 min and 40 min incubation in different media

(a) BHI

(b) MCDB 202

(c) D10



Images of samples (x100 immersion oil objective) stained with Nigrosin after cycled cells were transferred in to BHI (a), MCDB 202 (b) and D10 (c) broths. Samples were taken after 20 min (upper panels) and 40 min (lower panels). Cell were grown at 37 °C with shaking and stained with Nigrosin stain.

Figure 5.6 Nigrosin stain after 60 min and 24 h incubation in different media

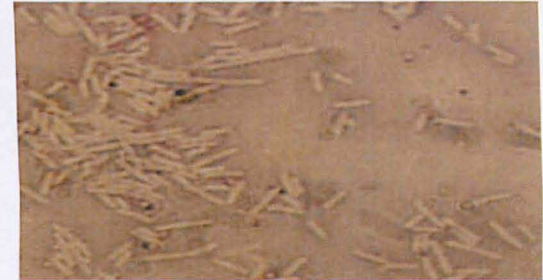
(b) BHI



(b) MCDB 202



(c) D10



Images of samples (x100 immersion oil objective) stained with Nigrosin after cycled cells were transferred in to BHI (a), MCDB 202 (b) and D10 (c) broths. Samples were taken after 60 min (upper panels) and 24 h (lower panels). Cells were grown at 37 °C with shaking.

The capsular material seen surrounding the *Listeria* cells was similar to results gained by Catchat *et al.* (2008) when they stained capsules produced by *Bacillus thuringiensis* strain BGSC 4AJ1 with Indian ink observed. Sue *et al.* (2006) also used Indian ink stains to observe capsules associated with *Bacillus cereus* responsible for causing severe pneumonia.

Capsule formation has been reported in most *Bacillus* strains including *Bacillus cereus* (Zhang *et al.*, 2009), *Bacillus anthracis* (Lee *et al.*, 2007), *B. thuringiensis* (Cachat *et al.*, 2008), *B. subtilis* (Moriakwa *et al.*, 2006) and *Bacillus licheniformis* (He *et al.*, 2000). It has also been reported in other Gram-positive bacteria such as *Staphylococcus epidermidis* (Kocianova *et al.*, 2005) and for a range of Gram-negative bacteria such as *Escherichia coli* (Sule *et al.*, 2009), *Pseudomonas aeruginosa* (Goldman *et al.*, 2009) and *Pseudomonas fluorescens* (Baum *et al.*, 2009). However, no phenotypic capsule production has previously been reported in *Listeria*.

The genus *Bacillus* is very close to *Listeria* in the phylogeny of organisms as deduced by comparison of rRNA sequences (Sallen *et al.* 1996). In fact, Glaser *et al.* (2001) suggested a common origin of *L. monocytogenes*, *B. subtilis* and *L. innocua* when sequence analysis of their genomes was carried out.

From the results of the Nigrosin capsule stain, there was clear evidence that the *Listeria* cells made capsular material but nature of the capsule was unclear. Since no report in literature has identified homologues of the sugar biosynthesis genes normally required for the synthesis of polysaccharide-based EPS material in the *Listeria* genome sequence, this genetic relationship between *Bacillus* and *Listeria*

perhaps could mean that the capsular material may be the same capsular material produced by *Bacillus*.

In the genome sequence of *L. monocytogenes* EGD-e Glaser *et al.* (2001) identified the gene *lmo0516* as being similar to the *B. anthracis* *capA* encapsulation gene required for capsule formation by this bacterium. Furthermore, Kocianova *et al.* (2005) found that *B. cereus* required only *capA* genes to make capsules. Begley *et al.* (2005) found that a transposon mutant in this *capA* homologue in *L. monocytogenes* LO28 had a bile-sensitive phenotype when screening mutant banks for bile resistance. Therefore, a hypothesis was formed that the extracellular polymeric substance forming the *Listeria* capsule was similar to the capsular material poly-gamma-glutamic acid (PGA) made by some species of *Bacillus*.

5.3 Giemsa capsule stain

Since the capsular material was presumed to be PGA, an experiment was designed to confirm the capsule stain results obtained using Nigrosin stain by staining the cells with Giemsa stain. Giemsa stain has been used widely in the identification of PGA capsules produced by *B. anthracis*. For instance the Giemsa capsule stain was used by Parkinson *et al.* (2003) to investigate an anthrax spore contamination in Alberta, Canada while Hawkes *et al.* (2008) used it to confirm the presence of *B. anthracis* capsules as a method of identification of the infectious agent in an infection of mixed breed beef cows.

The stain was carried out as described by Collins *et al.* (1995). Cells of strains Lm 23074, Lm 10403S, Lm 000101-0305, Lm 000622-0305, Lm 00048-0305, LO28 and Lm 00054-0305 were selected to represent the major serotypes that cause food borne Listeriosis.

To generate an inoculum, the different strains were prepared as in Section 3.4. Cells were also grown for 24 h and 10 days in BHI and D10 with shaking at 37 °C. This longer incubation was used to establish if cell age could affect production of capsular EPS. Staining was performed by fixing the cells with absolute methanol for 3 min. Giemsa stain was poured on the slides and left until almost dry before washing rapidly with RO water and then PBS (see Section 2.5).

The micrographs of the cells after 24 h of growth in the different media are shown in Figures 5.7-5.8. The presence of extra polymeric capsular material was detected surrounding cells grown in the defined media D10 and MCDB 202 (Fig. 5.7) indicated by the fact that the cells appeared exactly as described by Collins *et al* (1995), with the bacterial cells staining blue and capsules staining red or pink. In contrast cells grown in BHI had little observable capsular material (Fig. 5.7).

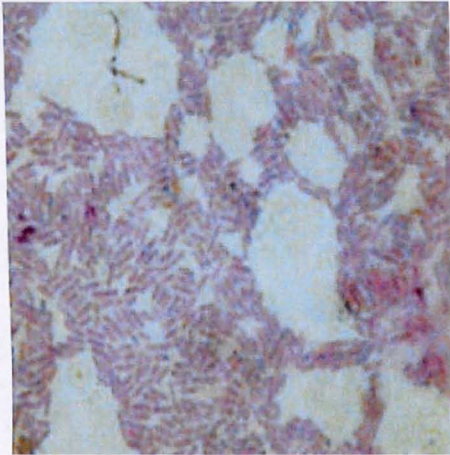
When incubated for a further 10 days (Fig. 5.8), both cells grown in BHI and cells grown in minimal D10 had detectable capsular material and wider areas of staining indicating that more EPS was formed in the older cultures. It also shows that the material was also made when the cells were grown in a rich media for long periods of time. However cells grown in minimal D10 for 10 days were less dispersed, and formed denser clumps than cells grown in BHI. This is similar to the results obtained using the Nigrosin stain and suggests that the capsular EPS material is more pronounced in cells grown in this more defined media, possibly because the cells enter a nutrient limited state before those grown in a rich media such as BHI.

Interestingly it has been shown by King *et al.* (2000) that *B. licheniformis* cells must be grown for three days in order to obtain a

good yield of PGA or EPS capsular material. However Troy (1973) found that during the early stage of cell growth polymers adhere to cells as true capsule. This suggests the capsule formation observed in the early growth stage in this study.

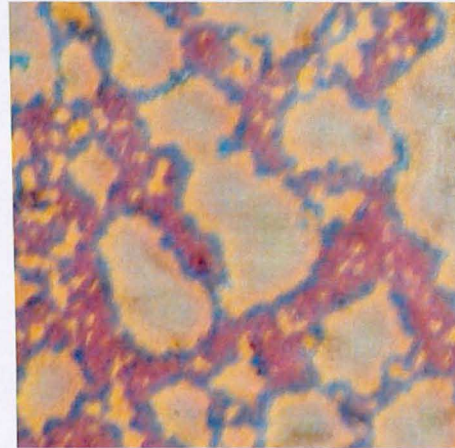
Figure 5.7 Giemsa stain of cells grown overnight in BHI, D10 and MCDB 202 media.

BHI



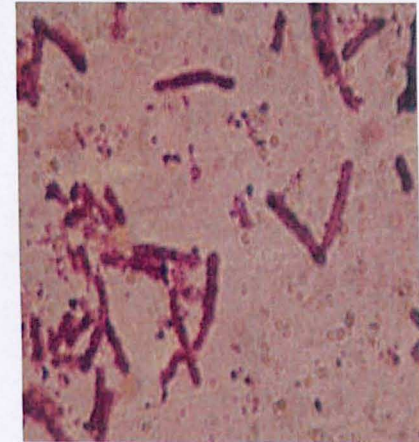
Lm 000101-0305

D10



Lm 10403S

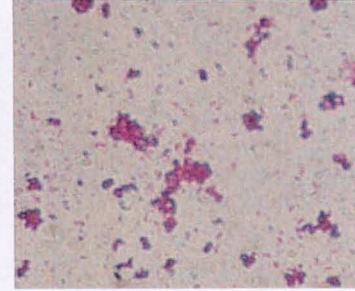
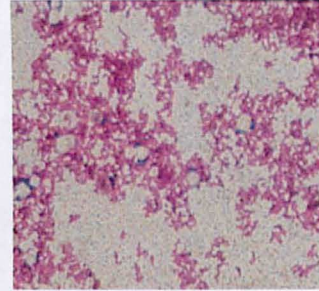
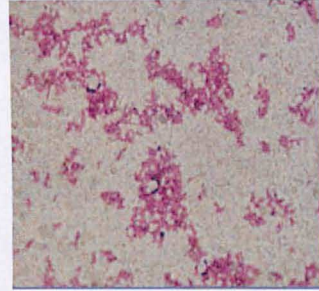
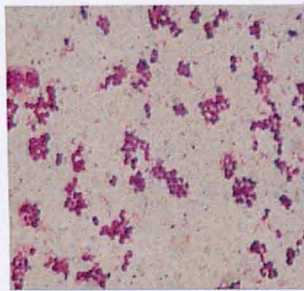
MCDB 202



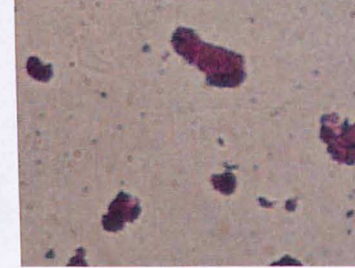
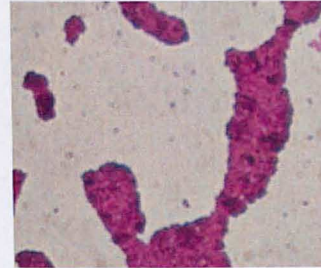
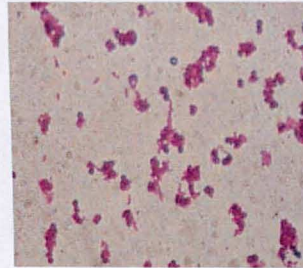
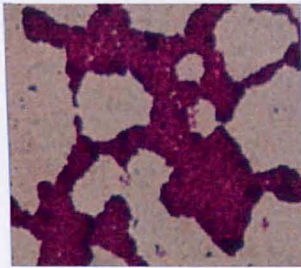
Lm 23074

Representative images (x100 immersion oil objective) of Giemsa stained *Listeria* cells grown in BHI, D10 and MCDB 202, respectively. Cells were grown for 24 h with shaking at 37 °C and stained with Giemsa stain.

Figure 5.8 Giemsa stain for cells grown in BHI for 10 days



BHI
D10



Lm 10403S

Lm 23074

Lm 00048-0305

Lm 000101-0305

Representative images (x100 immersion oil objective) of Giemsa stained *Listeria* cells grown in BHI for 10 days (top) and the corresponding strain grown in D10 (below). Cells in D10 had clearly detectable amounts of EPS.

5.4 Scanning Electron Microscopy of *Listeria* cells grown in different media

As the images gained under the light microscope did not give clear image of the capsular material it was necessary to examine the cells grown in different media with a higher resolving microscope. Scanning electron microscopy (SEM) was used because it has resolving power 50,000 times more than the light microscope and has previously been used to show detail of the surface of *Listeria* cells growing in biofilms (Chavant *et al.* 2007 and Rajeb *et al.* 2009).

The use of SEM on EPS produced by other organisms has also been carried out by other researchers. Liu *et al.* (2010) used SEM to study the release of EPS by bacteria during sludge dewatering and Olmez *et al.* (2010) used SEM to show effects of different sanitizing treatments on biofilms and attachment of *Escherichia coli* and *L. monocytogenes* on green leaf lettuce. Similarly using SEM, Wang *et al.* (2010) found that EPS may act as adhesive binding adjacent cells and thus further enhance the aggregation of microbes during corrosion of metals by marine bacterium *Vibrio natriengens*.

Cells of strain Lm 10403S were inoculated in 10 ml of BHI broth and grown at 37 °C with aeration overnight and prepared as in Section 3.4. The washed cells were inoculated into 10 ml of different media (BHI, D10, DJ+D10 and MCDB 202) and grown for 18 h before samples were viewed using SEM. To determine if EPS formation was species-specific, *L. innocua* was also grown in BHI and D10 media in the same way.

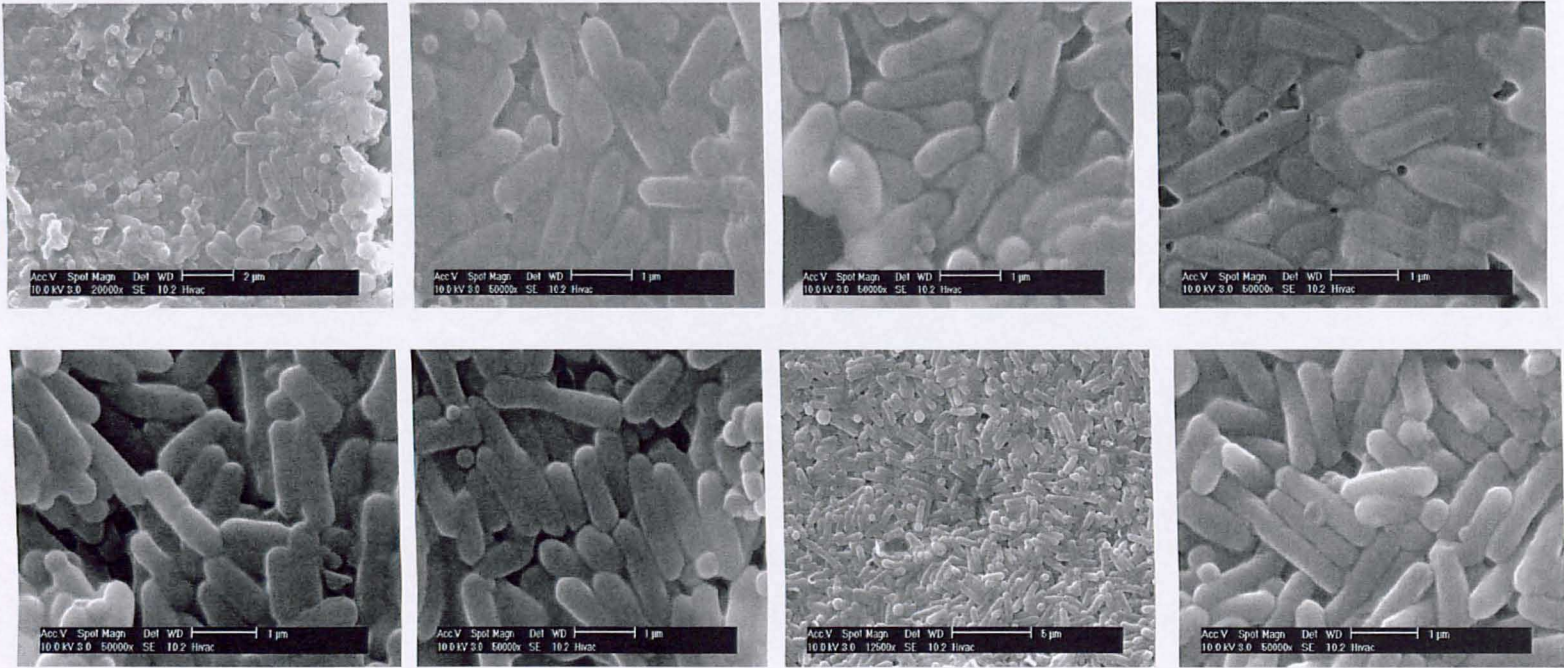
To prepare the cells for SEM, the cells were harvested from different media by centrifuging at 3000 x g for 15 min and were then fixed in acetone for 1 h (Section 2.16). A drop of cell/acetone mixture was placed on SEM stubs and allowed to dry, after which the stubs were

coated with platinum by sputtering. The stubs were finally viewed under high vacuum SEM.

The SEM images are shown in Figures 5.9-5.13. In BHI (Fig. 5.9) the planktonic cells appear as single rods with little evidence of EPS formation, although the outline of the cells is somewhat blurred suggesting there may be limited amounts of surface material being produced. However when grown in D10 media (Fig. 5.10) the single rod shaped cells had a visible coating of EPS material. At lower magnification (12500 x), the cells were seen to be covered by the EPS. At higher magnification (50000 X) visible EPS strings spanning between cells grown in D10 media were seen, indicating that the material was playing a role in holding the cells together (Fig. 5.10).

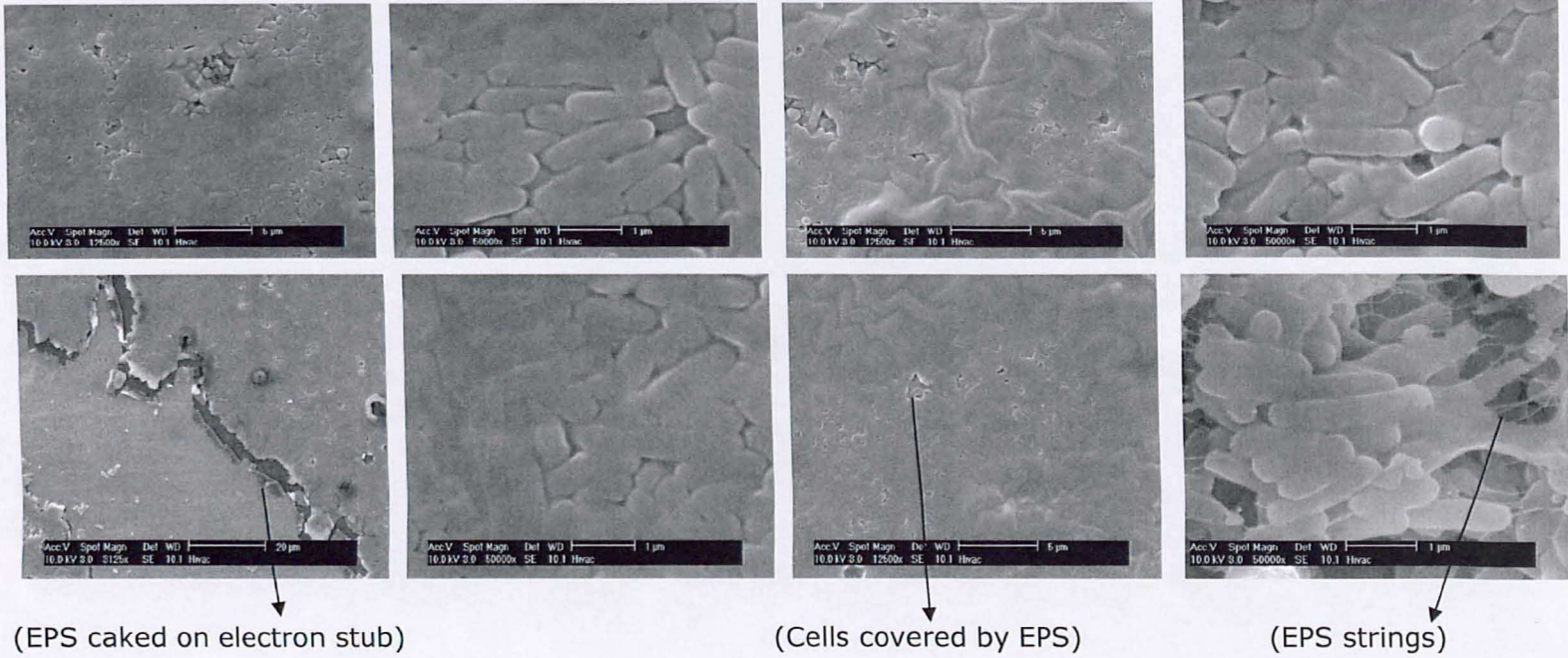
The same string-like structures were found on cells grown in MCDB202 although this had a more woolly quality (Fig. 5.11) while shorter strings were observed for cell grown in D10+DJ (Fig. 5.12). Hence it is clear that media composition influences EPS formation by *Listeria* with the most EPS being formed in minimal media.

Figure 5.9 SEM analysis of cells grown in BHI



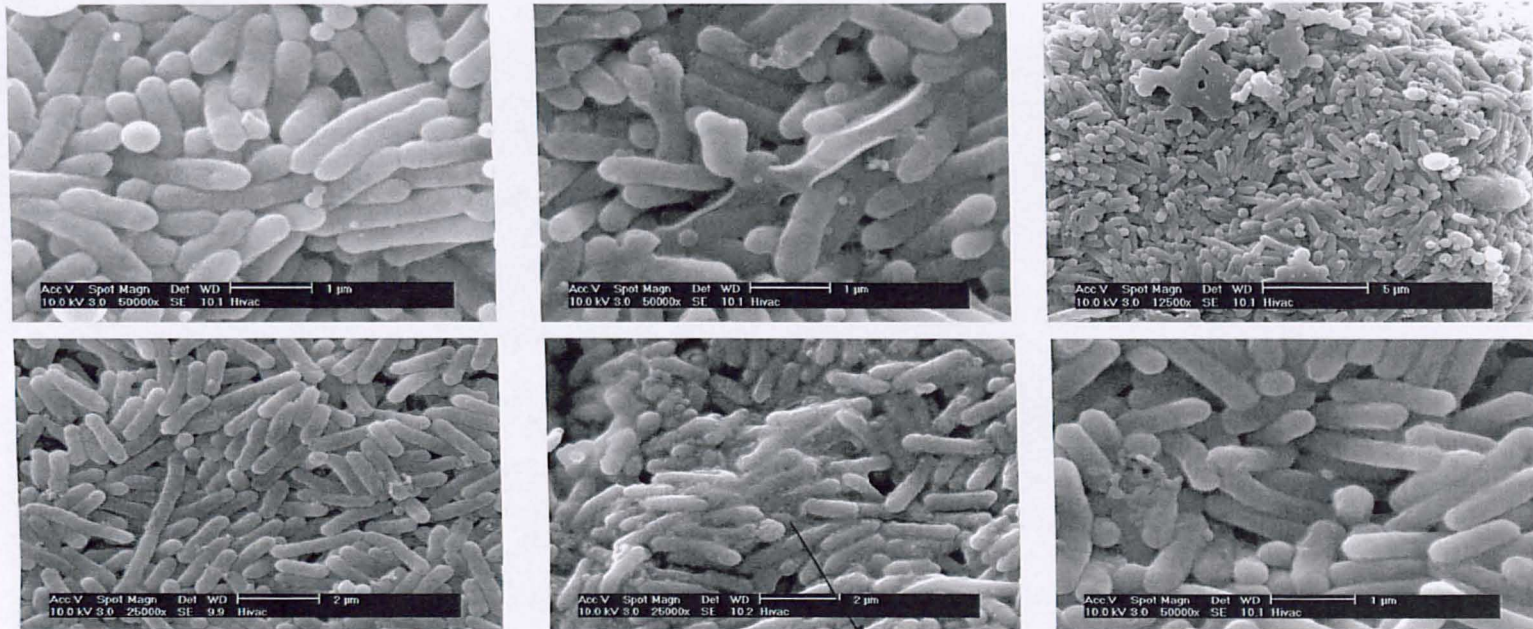
SEM micrographs for cells grown in BHI. Cells were grown over night with shaking at 37 °C and fixed on electron stubs before coating with platinum. Cells were viewed under high vacuum at a magnification of 50000 X.

Figure 5.10 SEM analyses of cells grown in D10



SEM micrographs for cells grown in D10. Cells were grown over night with shaking at 37 °C and fixed on electron stubs before coating with platinum. Cells were viewed under high vacuum at a magnification of 50000 X.

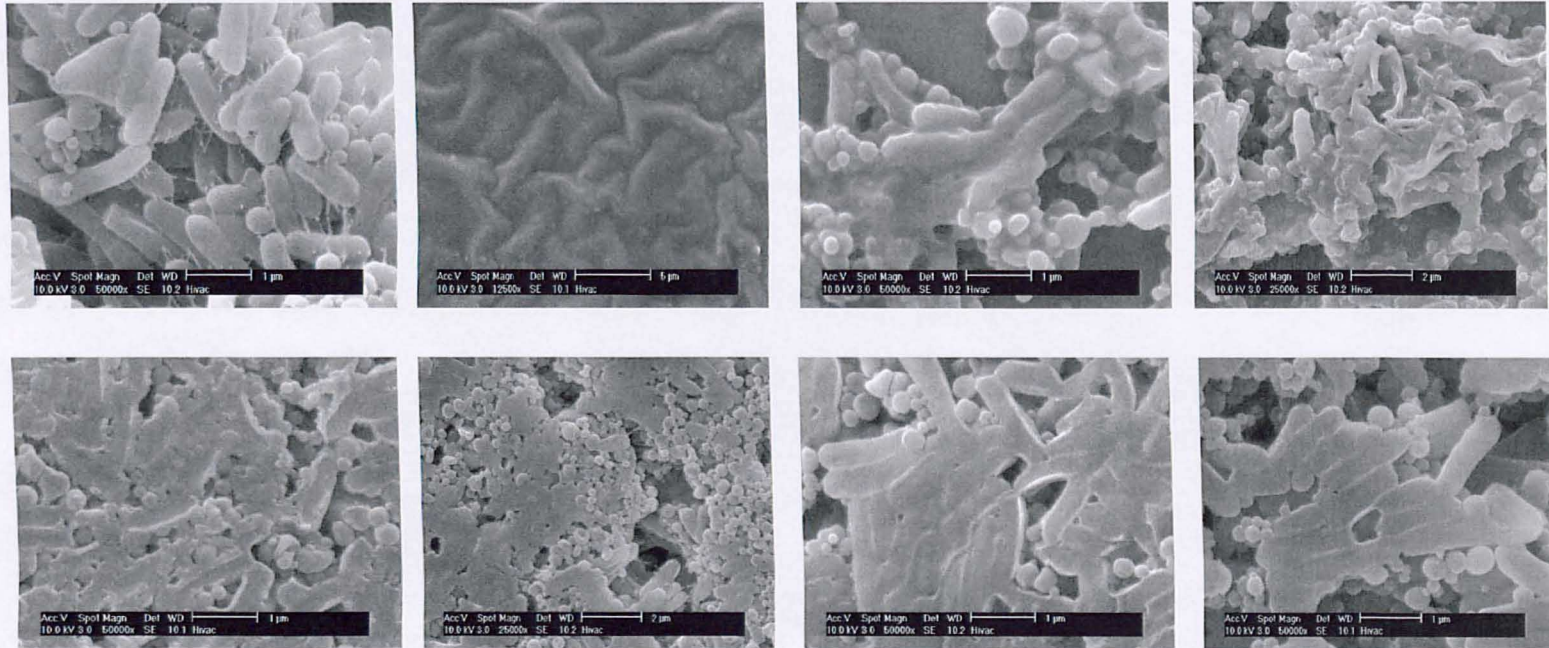
Figure 5.11 SEM analyses of cells grown in MCDB 202



Woolly-like EPS material covering the cells

SEM micrographs for cells grown in MCDB 202. Cells were grown over night with shaking at 37 °C and fixed on electron stubs before coating with platinum. Cells were viewed under high vacuum at a magnification of 50000 X.

Figure 5.12 SEM analyses of cells grown in DJ+D10

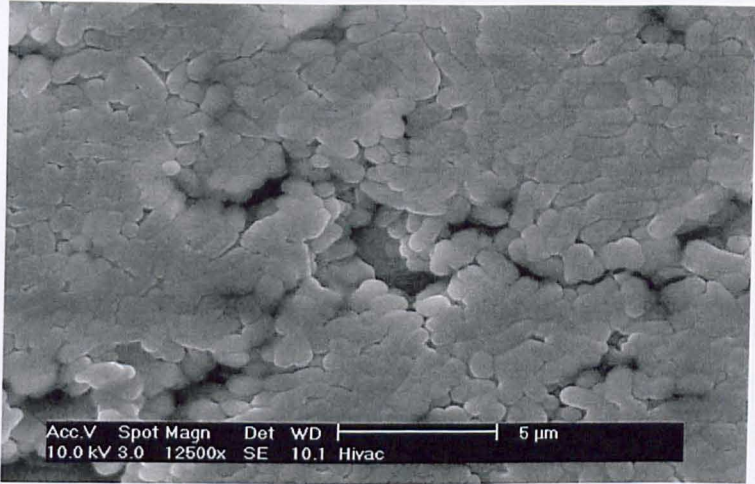


SEM micrographs for cells grown in DJ+D10. Cells were grown over night with shaking at 37 °C and fixed on electron stubs before coating with platinum. Cells were viewed under high vacuum at a magnification of 50000 X.

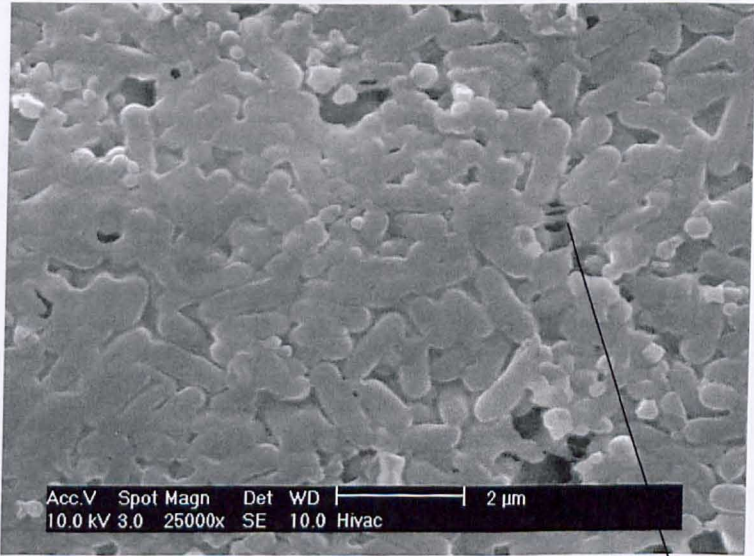
The non-pathogenic *L. innocua* also showed the presence of EPS strings when grown in D10 (Fig. 5.13) which was less pronounced for cells grown in BHI, although the smoothing of the cell surface did suggest that some EPS may be being produced (Fig. 5.13). For *L. innocua* the EPS strings appeared shorter in than those seen in the *L. monocytogenes* samples but these results indicate that both virulent and non-virulent *Listeria* species can make EPS.

Figure 5.13 SEM of *Listeria innocua* grown in different media

(a)



(b)



String like EPS

Scanning electron micro graphs of cells of *L. innocua* grown for 18 h in BHI (a) and D10 (b), dried in acetone and coated with platinum. Strings of EPS showing in D10 were absent in BHI.

Many workers have visualized EPS of *Listeria* but it has always been biofilm images on solid surfaces i.e. Biofilm/EPS matrix (see Sandasi *et al.*, 2008; Rieu *et al.*, 2008 and Olmez and Temur, 2010). However this is the first evidence of EPS being produced during liquid growth of cells.

The microscopy analysis indicated that cells grown in minimal media had more EPS, correlating with hydrophobicity results which indicated that BHI cells clearly had different surface properties and were partitioned to a lesser extent into solvents. Also some differences in structure (presence of strings) seen between cells grown in D10 and MCDB202 EPS could explain differences in behaviour seen between MCDB 202 and D10 in MATH assays described in Chapter 4. Having established the presence of EPS in *Listeria* cells, it was now necessary to purify the material and investigate its composition.

5.5 Conclusion

The staining methods used (Giemsa and Nigrosin) showed that *L. monocytogenes* produced extracellular polymeric capsular material, and that this was produced in greatest amounts when the cells were grown in minimal media or underwent prolonged culture in rich broth and capsule production seemed to be associated with the clumping or sticking together of cells.

1. Capsular material production began almost immediately with cell growth and was not nutrient limiting. There was evidence that the EPS was made by planktonic cells, especially in minimal media.
2. The SEM analysis proved that the EPS plays a role in holding the cells together.
3. Extracellular polymeric capsular materials are formed in both virulent and non-virulent *Listeria* species.

Chapter 6 RESULTS:

Purification and initial characterisation of extra cellular polymeric substance

6.1 Extraction and purification of *Listeria* polymer

Having established that indeed EPS was being formed it was now necessary to isolate the EPS to allow further characterisation. To this end the EPS capsular material was extracted and purified as described by Goto and Kunioka (1992), Kambourova *et al* (2001) and Cachat *et al* (2008). Their method was chosen because it has been used to extract capsular material PGA from *Bacillus* and it was suspected that the *Listeria* EPS material might be similar in nature.

For the purification, D10 media was chosen because SEM analysis and Nigrosin staining had showed that the formation of the EPS was most pronounced in D10 media. The strain Lm 10403S was used because it stained very well for EPS using Nigrosin and Giemsa stain in addition to the visual observations made during the SEM analysis

As before cells of Lm 10403S were prepared as in section 3.4 before resuspending the cells in 50 ml of D10 broth in a 100 ml conical flask. The cells were grown with shaking at 37 °C for two days and capsule production was checked at the end of growth period by performing a Nigrosin capsule stain of samples taken from the culture suspension.

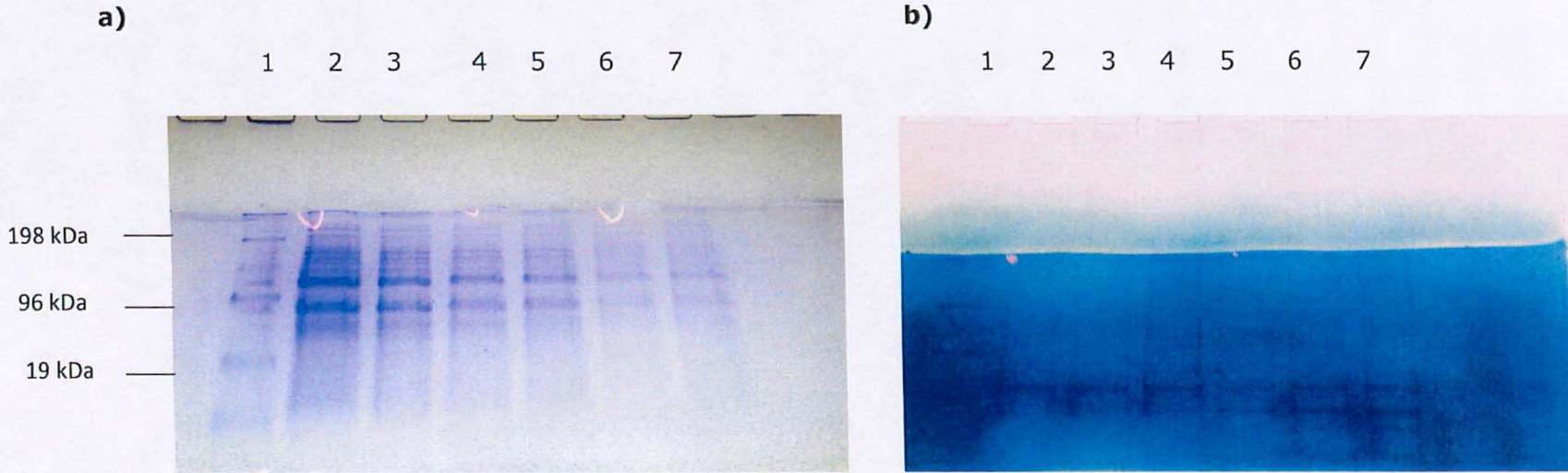
To extract the EPS, glass beads 3mm in diameter (0.3 g) were added to an equal volume of culture broth and ice and vortexed for 2 min. The vortexed culture was decanted into centrifuge tubes and centrifuged at 15000 x g at 4 °C for 10 min after which the supernatant was collected. Four volumes of previously cooled ethanol cooled (-20 °C) were added to the supernatant and this was left to allow precipitation to occur for 24 h at -20 °C.

The supernatant containing the precipitates was centrifuged at 5000 x g for 5 min to recover the pellets. The pellets were dissolved in 20 volumes of distilled water and lyophilized under vacuum at -85 °C overnight to produce crude crystals. The crude crystals were re-suspended in 20 volumes of distilled water and centrifuged for 15 min at 20000 x g at 4 °C. The supernatant was dialyzed overnight at 4 °C using distilled water and this was then lyophilized after to produce the pure EPS material which was reconstituted at a concentration of 5 mg/ml.

To determine if the extraction was successful and to obtain an estimate of the molecular weight, the purified material was subjected to analysis by SDS-PAGE as described by Kambourova *et al.* (2001) who used SDS-PAGE to visualise purified capsular material extracted from *Bacillus*. After preparing the gels, 5 µl of the EPS suspension were loaded onto wells along with protein standards and the samples electrophoresed for before 3 h. The gel was then stained with Coomassie blue before destaining and then stained with methylene blue (Catchat *et al.*, 2008).

The results of the SDS-PAGE analysis are shown in Figure 6.1. The proteins associated with the purified EPS material were visible after staining with Coomassie blue (Fig. 6.1a) but there was no visible evidence of the polymer in the stacking area where it was expected that the material would be. However after staining with methylene blue the polymer was detected in the stacking area (Fig 6.1b). This result was consistent with the hypothesis that the EPS material was similar to *Bacillus* PGA. .

Fig 6.1 SDS analysis of *Listeria* EPS using Coomassie blue and methylene blue stain



Visualisation of the EPS using SDS-PAGE in a 10% gel. Two gels were prepared and one was then stained with Coomassie blue (panel a) and the other with methylene blue (panel b). Purified crystals were used at 5mg/ml . In gel A the stacking area showed no staining with Coomassie blue while in gel B the stacking area was stained by methylene blue. Lanes 2-7= *Listeria*EPS. Samples were duplicated six times.

6.1.1 Removal of cell associated proteins of *Listeria* EPS with pronase

The associated proteins detected by SDS-PAGE analysis indicated that the EPS material needed to be purified further. This was necessary to prove that the EPS was not composed of protein. Also the EPS of *Bacillus* was shown by Catchat *et al.* (2008) to be resistant to pronase. The purification was carried out by treating the EPS with pronase. A 5 μ l of the pronase enzyme (20mg/ml) was placed in 5 ml of the *Listeria* EPS suspension and incubated at 42 °C for 18 h.

Following pronase treatment the pronase enzyme was removed from the capsular material by filtered through a 100,000 mwt cutoff filter. To do this a 500 μ l of (5 mg/ml) sample of the reconstituted pronase-treated material was placed in a filter unit and centrifuged for 12 min at 500 x g. The retentate was recovered and lyophilized (section 2.17). To verify that the associated proteins have been removed from the EPS material, the purified material was subjected again to SDS-PAGE and samples that had not undergone pronase treatments were used as control. After pronase treatment there was no staining of the polymer with Coomassie blue while the untreated samples still produced the same pattern when stained (Fig. 6.2a).

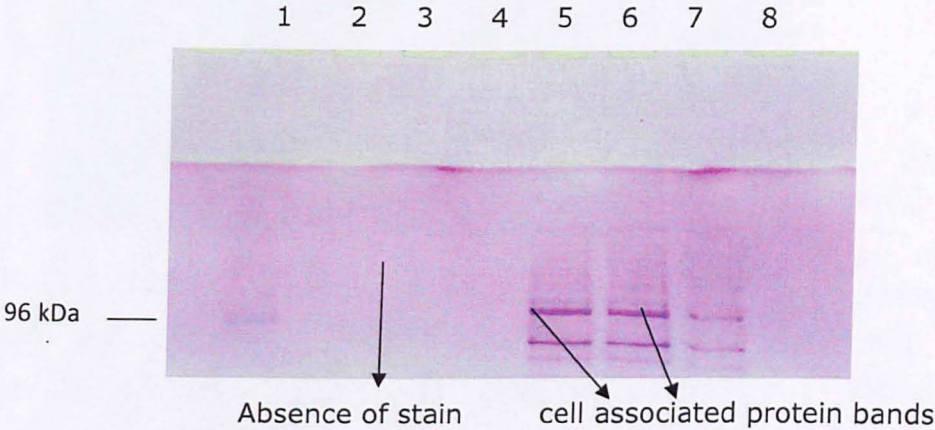
When stained with methylene blue (Fig. 6.2b) both treated and untreated samples stained indicating that the *Listeria* EPS is methylene blue sensitive and not Coomassie sensitive just like PGA. The EPS material that stained with methylene blue (Fig. 6.2b) had no protein bands in it confirming that the proteins in this sample were removed by the pronase treatment.

When the concentration of the EPS was increased to 10 mg/ml before loading, bands formed more clearly in the stacking area (Fig. 6.3) instead of appearing as long smears. This was taken to indicate that

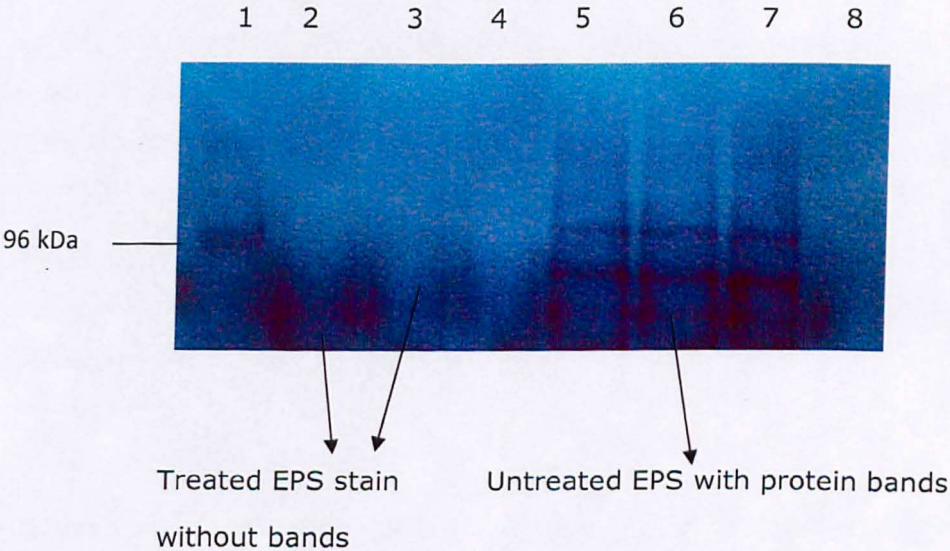
the material is of a high molecular weight since the material did not migrate out of the stacking area along with the proteins.

Figure 6.2 Effect of pronase treatment

A) Coomassie blue stain

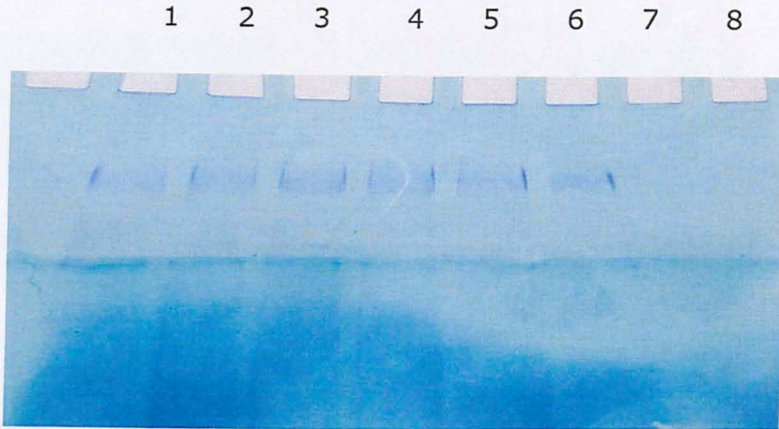


B) Methylene blue stain



SDS-PAGE in a 10% gel of EPS material after treatment with pronase. In panel A and B; Lane 1= molecular weight markers; lanes 2-4, EPS treated with pronase (three replicates); lanes 5-7, untreated EPS samples (three replicates). Lane 8 was blank without sample to serve as negative control.

Figure 6.3 SDS-PAGE analysis with increased concentration



Increased concentration of *Listeria* EPS material (10 mg/ml) stained with methylene blue (lanes 1-6) i.e six replicates showing in the stacking area of the SDS -PAGE gel (10%) run for 3 h. Lanes 7 and 8 contained no samples to serve as control.

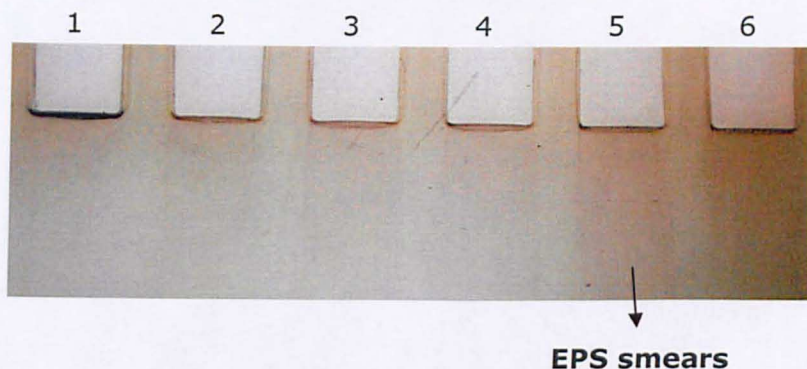
6.2 Silver stain of *Listeria* EPS

In order to detect the presence of the EPS more clearly a more sensitive stain was used. Although silver stain is mainly used for proteins (Merill *et al.*, 1981), it has found application in the detection of small amounts of other organic compounds (Lambert *et al.* 2009).

A fresh gel was prepared, fixed and soaked in the solutions provided in a Silver Staining kit (GE Healthcare; see section 2.18). Finally, the gel was developed for approximately 6 min.

The stain proved positive since brown colouration of the EPS material in different lane was observed indicating that the EPS was present.

Figure 6.4 Silver stain of EPS material



Silver stain of *Listeria* EPS in a 10% SDS-PSGE gel. Lane 1= control without sample, Lanes 2-6= EPS material. There was brown staining or colouration of the EPS with silver stain. Each track was loaded with *Listeria* polymer at a concentration of 5mg/ml.

6.3 Conclusion

The purification method used was successful and the material purified was detected using both, methylene blue and silver staining. The study showed that the *Listeria* EPS was staining with methylene blue but did not stain with Coomassie blue and is also resistant to protease treatment, confirming that the material is not composed of protein. The fact that the EPS was retained in the stacking area of the SDS-PAGE gels indicates that the material is of high molecular weight. However, although the results to date were consistent with the material being similar to *Bacillus* PGA, these analysis did not give any more information about the specific chemical nature of the material and hence further characterization of the EPS was necessary.

Chapter 7 Results:

Characterization of extracellular polymeric substance (EPS) from *L. monocytogenes*

7.1 Introduction

After observing EPS formation and accumulating some data regarding its physical properties, it was now very important to characterize and determine the nature and composition or actual chemical structure of the *Listeria* polymer so that the genes involved in its synthesis can be identified, and hence lead to a better understanding of the regulation of the synthesis of this polymer. This is particularly important since EPS production has been linked to persistence in factories by facilitating initial attachment and formation of biofilms in other organisms.

Neu and Lawrence (1999) described how EPS analysis can be performed either destructively or non-destructively. Destructive analysis includes isolation by precipitation, purification by size fractionation and analysis of EPS constituents by high performance liquid chromatography and gas chromatography and isolation of oligosaccharides to confirm structure. In this study, no isolation of oligosaccharides is expected because *Listeria* does not have homologues of the genes required to make capsules composed of sugar. If the EPS material has a pure ordered form the crystalline structure may be investigated using X-ray powder diffraction (Blow, 2002).

Non-destructive analysis that can be undertaken includes Attenuated Total Reflectance - Fourier Transformed Infrared (ATR-FTIR) spectroscopy and Nuclear Magnetic Resonance (NMR). The basis of NMR is that the resonance frequency of a particular compound is directly proportional to the strength of the applied magnetic field. By examining the peaks of a nuclear magnetic spectrum of a given compound, it can be identified by comparison with peaks of a known compound.

The FTIR measures the wavelength and intensity of the absorption of mid-infrared light by a sample over time. The wavelength of infrared absorption bands can be measured and are characteristic for a

particular bond. This technique is particularly good for organic molecules in a complex matrix.

Previous attempts have been made to characterize and identify the EPS constituents of *Listeria* but the attempts have not been holistic as previous workers focused on or highlighted some constituents of *Listeria* EPS. Harmsen *et al.* (2010) reported that extracellular DNA is the only central component of *Listeria* EPS. It has also been reported that biofilms of *Listeria* contain carbohydrates (Boruki *et al.*, 2003; Chae *et al.*, 2006; Zameer *et al.*, 2010) since *Listeria* biofilms were stained with ruthenium dye. Ruthenium dye is a cationic stain initially used by botanists as a semi specific stain for pectic substances, but it has gradually been embraced by investigators in microbiology and the animal sciences as a stain for anionic glycosylated polymeric substances (Fassel and Edmiston, 1999).

Hefford *et al.* (2005) also showed the presence of carbohydrate in biofilms of *Listeria* by labeling adherent layers with Fluorescein-conjugated concanavalin A which binds specifically to alphanmannosyl groups found in sugars, glycoprotein and glycolipids. In addition they identified nineteen proteins being present in their study.

Other workers utilized phenol (Singh *et al.*, 1981) and saline extractions (Otokunefor and Galsworthy, 1982) of *Listeria* EPS which was found to contain carbohydrates, amino acids, and phosphorous. Hether and Jackson (1983) also used phenol extraction and reported the compound extracted was Lipoteichoic acid (LTA). The LTA had amphiphilic properties and contained glucose, galactose, fatty acids, glycerol and phosphate with molar ratio of 0.05, 0.07, 0.21 and 0.94 and 1.0 molar ratio to phosphates.

The conflicting results gained from all these studies indicate that despite all these efforts, no one report has comprehensively established the nature of the major polymers present in *Listeria* EPS.

To perform the characterization of the *Listeria* EPS, several methods were chosen to investigate the nature of the polymer purified in this work. These included SEM and elemental analysis, sugar and amino acid analysis, liquid and solid state (NMR), X-ray diffraction (XRD) and size exclusion multi angle laser light scattering (SEC-MALLS).

7.2 Scanning electron microscopy and elemental analysis of purified EPS of *Listeria monocytogenes*

SEM was carried out to view, in detail, the purified *Listeria* polymer separate from the *Listeria* cells. The analysis was performed at the school of manufacturing engineering with the assistance of Nicola Weston. A small amount (1 µg) of the purified extract which was treated with pronase (section 2.16) was placed on the SEM stubs and then coated with platinum by spurting (Polaron SC7640) for 90s.

The stubs were finally viewed under high vacuum. After capturing images in dry mode under high vacuum, the machine was switched to elemental analysis mode to capture the elemental components from three different randomly selected surface areas containing the dry material. One (4th measurement) area where there was no material detectable on the stubs served as control.

After this, the vacuum was removed and moisture was then applied to the dry material gradually. Images were captured to monitor the rehydration process as relative humidity increased. Moisture was applied automatically at different vapor pressures (Torr) and images of rehydrated material at different relative humidities were captured. After rehydration, elemental analysis was again performed on the purified material.

Figure 7.1 shows images of the dry EPS material. The structure was generally irregular and there was absence of *Listeria* cells observed in the SEM of EPS matrix seen in Chapter 4. This showed that the cells were properly separated from the EPS. Swelling was observed when the dry material was rehydrated (Fig. 7.2). The rehydration process

showed rapid absorption of water by the EPS with increase in water absorbance as relative humidity increased. From the first monitoring at 25% relative humidity (Fig. 7.3), the polymer absorbed water and swelling was quite noticeable at 50% relative humidity. Swelling was prominent at 65% relative humidity and the compound was completely saturated, visually, at 100% RH.

After rehydration, the EPS retained water and was still wet after the stubs were left overnight at room temperature showing that it was able to retain or store water for long time periods.

Elemental analysis of the dry (Fig. 7.4) and wet (Fig. 7.5) EPS revealed a similarity in elements detected. The most abundant was carbon and oxygen with no nitrogen peaks observed. However, the carbon peak reduced dramatically when areas free from the material on the stub was subjected to elemental analyses under both dry (Fig. 7.3d) and wet (Fig. 7.4d) conditions. This shows that the EPS material contains carbon in high amounts.

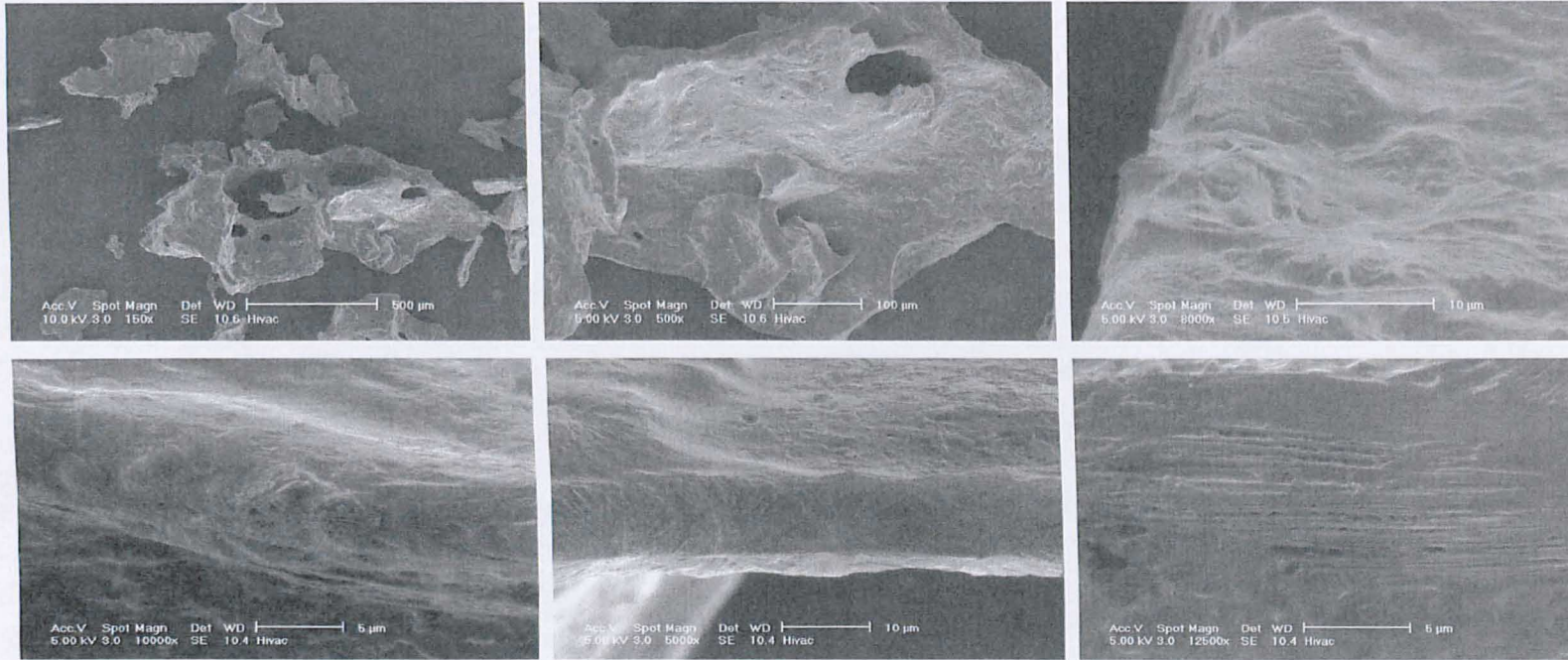
Other elements seen include sodium and phosphorous. Also calcium was found on the fourth or control area analyzed (Fig. 7.3d and 7.4d) but no calcium was observed from other three areas on the stub that had a lot of the EPS material (Figs 7.3 a-c and Figs 7.4 a-c). This shows that calcium may not be part of the material.

In Figure 7.4c the potassium (k) content diminished when rehydrated indicating that the potassium is solubilized. Also, the platinum that was used to coat the electron stubs showed as a constituent in the dry materials but was not detectable in the wet samples. Again this is probably because the potassium dissolved into solution.

The EPS may not be amino acid-based due to the lack of nitrogen which contradicts the findings of Harmsen *et al.* (2010) who reported that the dominant EPS material in *L. monocytogenes* is DNA. This is because bases in DNA contain a significant amount of nitrogen. Hence

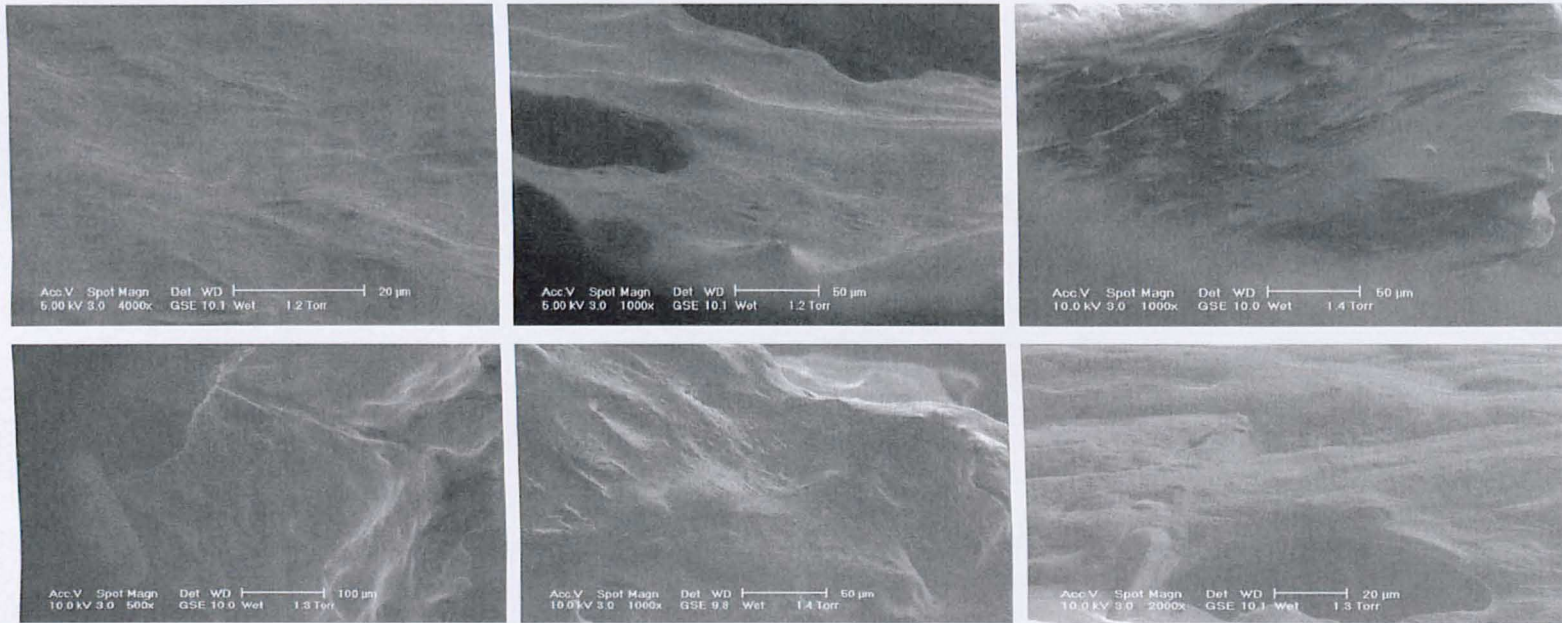
a result of no nitrogen or little amount of it indicates that DNA may not be the dominant material.

Figure 7.1 SEM of dry EPS



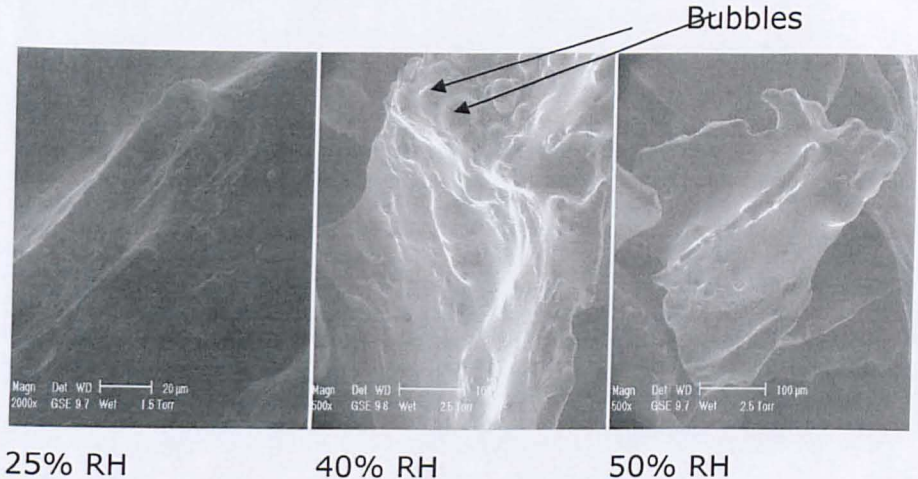
Representative images of purified dry *Listeria* EPS fixed on electron microscopy stubs and examined under high vacuum. There was absence of *Listeria* cells and the structure was irregular.

Figure 7.2 SEM of rehydrated EPS

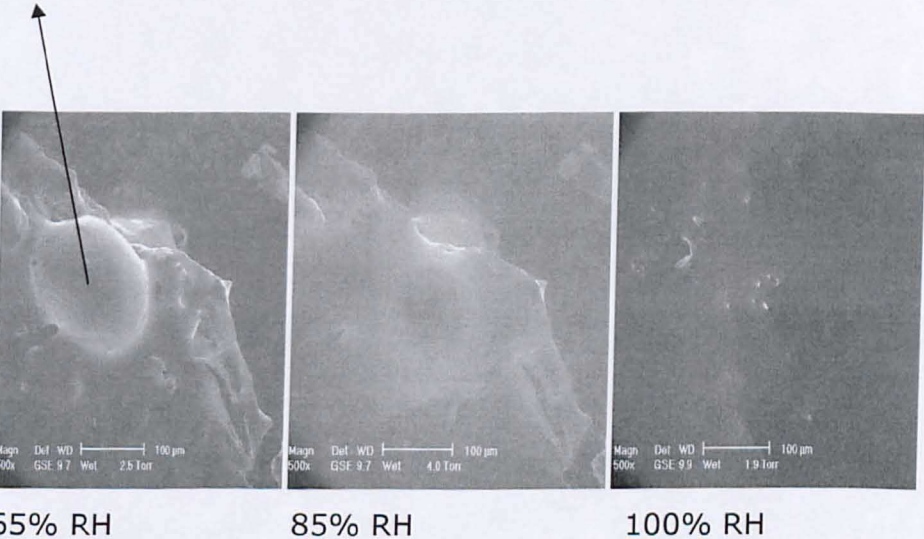


Representative images of purified EPS examined under wet mode without high vacuum. There was absence of *Listeria* cells and the crystals took on dough like appearance.

Figure 7.3 Effects of increasing Relative Humidity during rehydration of dry EPS

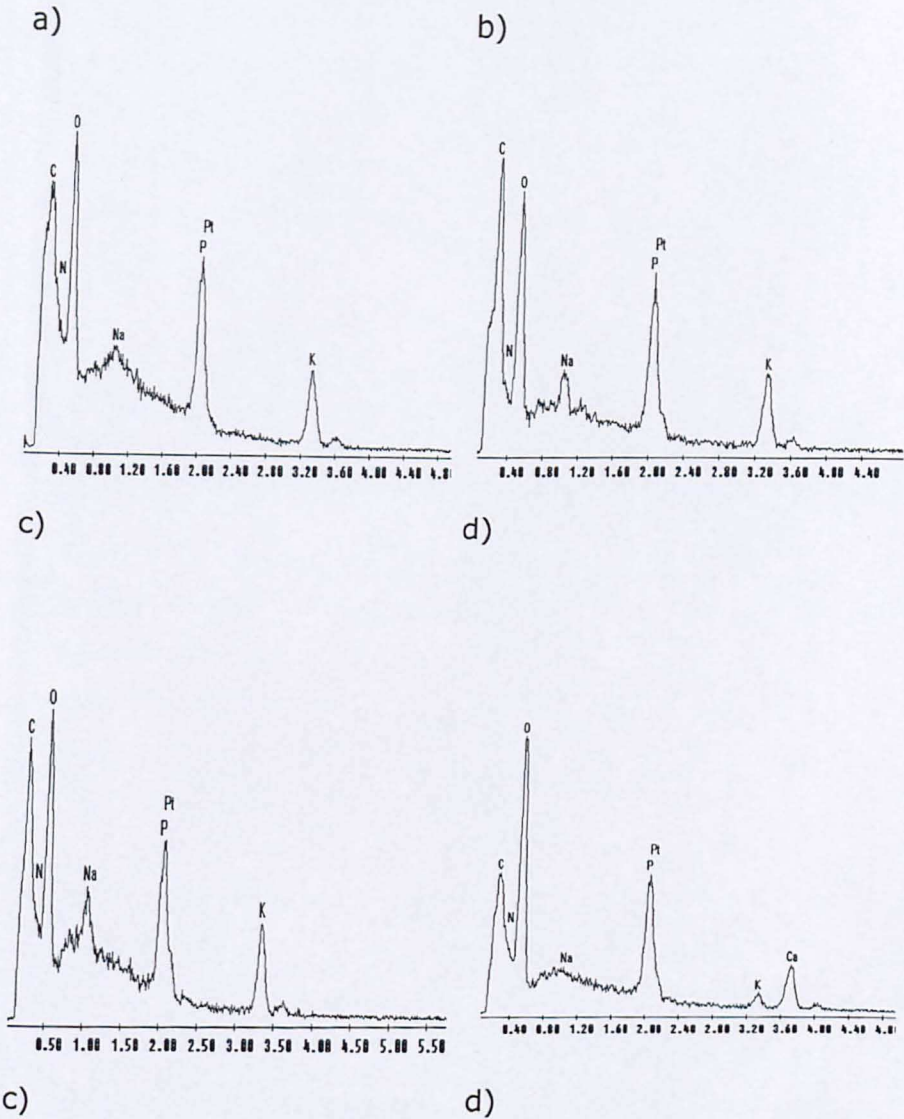


Swelling



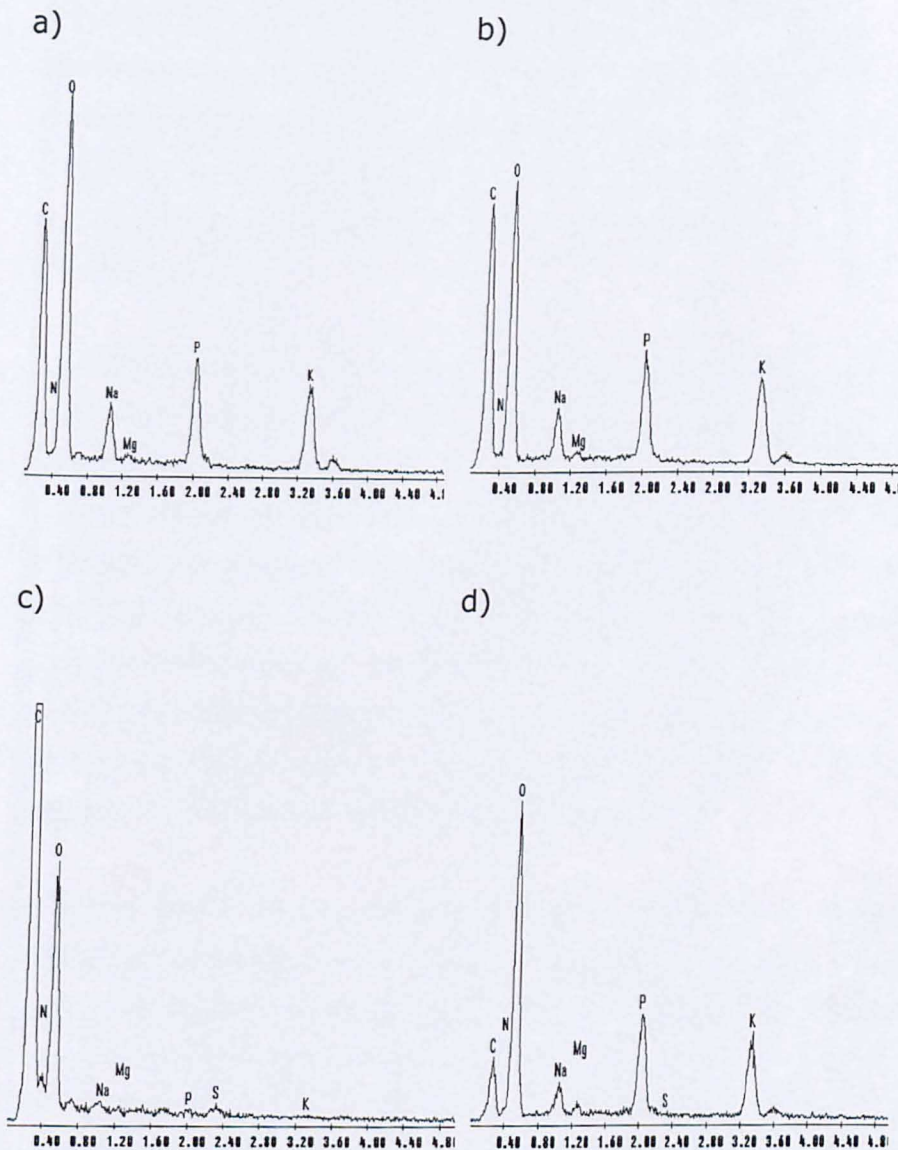
Representative images of purified EPS during the rehydration process of the dry polymer at different relative humidities (RH). Bubbles formed indicates an element of surface activity.

Figure 7.4 Elemental analysis of dry EPS



Elemental analysis of dry EPS. Panels a – d show the results of the analysis of four different spots on the surface of the EM stubs. The results in each case showed that carbon, oxygen, sodium, phosphorous and potassium were the predominant elements present. Carbon and oxygen were the most abundant and no particular peak could be seen in the Nitrogen region. Panel (d) shows the results obtained when an area with no visible material was sampled and was low in carbon.

Figure 7.5 Elemental analysis of rehydrated EPS



Elemental analysis of wet EPS. Panels a – d show the results of the analysis of four different spots on the surface of the EM stubs. Again this showed that carbon, oxygen, sodium, phosphorous and potassium were the most abundant elements and the levels Nitrogen were very low. Again carbon and oxygen were the most abundant with absence of nitrogen. Panel (d) shows the results obtained when an area with no visible material was sampled and was low in carbon.

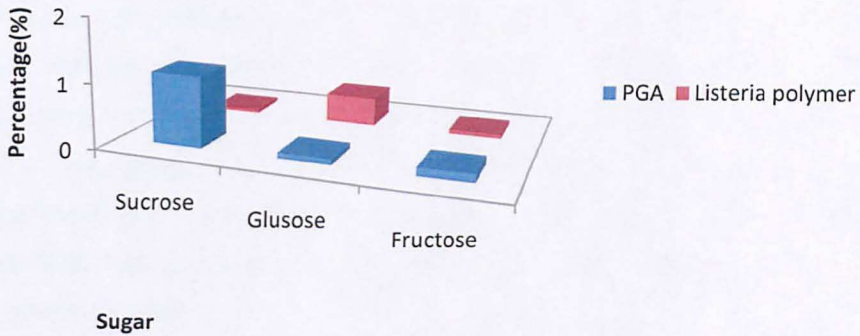
7.3 Sugar Analysis of purified EPS of *Listeria monocytogenes*

Since many bacterial capsular structures are made of sugar, and the elemental analysis indicated that the major elements present were carbon and oxygen, sugar analysis was carried out. As it had originally been suspected that the EPS may be similar to the *Bacillus* PGA, purified cosmetic grade PGA polymer (Vedan; Taiwan) was included in the analysis.

The analysis was carried out at the Division of Nutritional Sciences with the help of Gillian West. To carry out the sugar analysis, purified polymer treated with pronase (section 2.17) underwent acid hydrolysis with 1M H₂SO₄ at 100 °C to release any monomeric sugars and sugar analysis was carried out using UV analysis using the Sucrose/D-glucose/D-fructose kit (section 2.19). This kit works by measuring the nicotinamide-adenine dinucleotide phosphate (NADPH) released during the enzymatic hydrolysis of the sugar in question. The NADPH formed is stoichiometric to the amount of sugar present and is measured by means of its absorbance at 340 nm.

In both cases the quantities of sugar detected were very low, suggesting they were minor contaminants of the material rather than major constituents of the polymers. The PGA contained more sucrose and fructose than the *Listeria* EPS (Fig. 7.6) while *Listeria* EPS had more glucose. This result provides more evidence that PGA and *Listeria* EPS are not the same material. The fact that this polymer does not appear to be sugar-based is as expected since *Listeria* is not known to contain the genes required for polysaccharide synthesis.

Figure 7.6 Sugar analysis of *Listeria* EPS and PGA



After acid hydrolysis with 1M H_2SO_4 , very little complex sugar was detected. Sugar analysis of *Listeria* EPS and PGA showed that there was more sucrose in PGA while the *Listeria* EPS had more glucose, but in both cases these seem to be minor components of the purified material. The commercial kit had standard material for assay control purposes and kit used can only assay for sucrose, glucose and fructose.

Chae *et al.* (2006) reported that there were significantly high level of carbohydrates or polysaccharides in *Listeria* biofilms and these polysaccharides were detected at higher levels in attached cells than was found associated with planktonic cells, although the actual values they reported were very low (0.035-0.154 $\mu g/\log_{10}cfu$ free glucose and 1.71-9.45 $\mu g/\log_{10}cfu$ for total carbohydrates). If converted to percentage, comparing the upper and lower values quoted, the free glucose levels were 1.6-2% for lower and upper range values, respectively. There levels are consistent with the trace amounts of sugar detected in our purified material and suggest that sugars are not in fact a major component of this EPS material.

7.4 Amino acid analysis of purified EPS of *Listeria monocytogenes*

Again, as it had been proposed that the *Listeria* EPS could be PGA or a similar amino acid-based polymer, amino acid analysis was carried out and the results compared to the amino acid content of PGA. The analysis was carried out at the Division of Animal Sciences with the help of Li Dongfang. It was performed with an amino acid analyzer and approximately 300 mg of dry sample which has not been treated with pronase was processed (see section 2.20) before loading onto the amino acid analyzer.

Table 7.1 shows the amounts of amino acids detected in the polymer. Overall these were very low with arginine being detected in the greatest amount, but this represented only 0.4 % (w/w) of the total weight of the material. In contrast, and as expected, glutamic acid was the dominant amino acid in PGA but even this represented only 20% of the material indicating that this was not a highly purified sample and that other substances must be present contributing 80% of the total mass.

While the *Listeria* polymer contained traces of 16 amino acids, the PGA had only six. However the most important finding is the absence of significant amounts of glutamic acid in the *Listeria* EPS indicates that this polymer is not infact PGA. The absence of any significant amount of amino acids in the *Listeria* polymer also suggests that it is not a polymer made from another amino acid and agrees with the results of the elemental analysis presented in section 7.2, where the absence of the Nitrogen peak was noted.

Table 7.1 Amino acid composition of purified *Listeria* EPS

| Amino Acid Polymer | <i>Listeria</i> EPS (g/kg) | PGA (g/kg) |
|-----------------------|-------------------------------|---------------|
| Cystine | 0.88 | 0.09 |
| Aspartic acid | 1.84 | 0.34 |
| Methionine | 0.38 | 0.00 |
| Threonine | 1.10 | 0.00 |
| Serine | 1.10 | 0.00 |
| Glutamic acid | 3.10 | 193.22 |
| Glycine | 1.72 | 0.07 |
| Alanine | 1.77 | 0.00 |
| Valine | 1.07 | 0.00 |
| Isoleucine | 1.09 | 0.10 |
| Leucine | 1.15 | 0.00 |
| Tyrosine | 1.26 | 0.00 |
| Phenylalanine | 1.24 | 0.04 |
| Lysine | 2.06 | 0.05 |
| Histidine | 0.31 | 0.00 |
| Arginine | 3.69 | 0.00 |

Amino acid content of *Listeria* EPS without pronase treatment compared with the amino acid content of PGA.

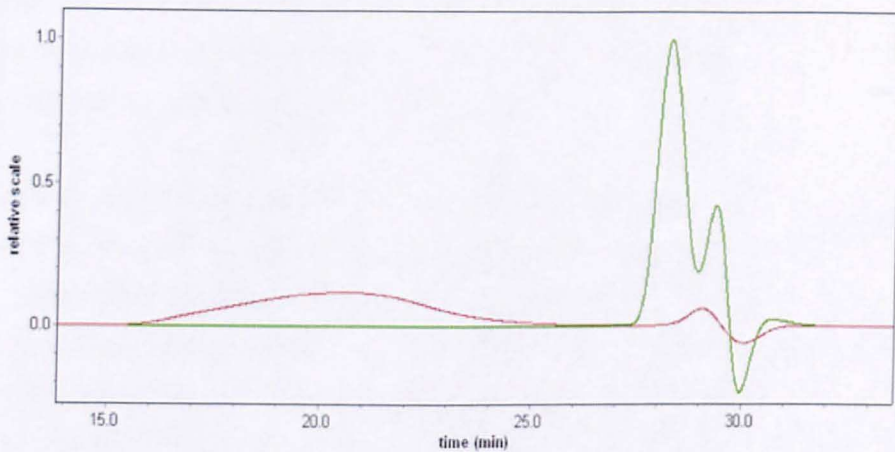
7.5 Size exclusion chromatography coupled to multi-angle laser light scattering (SEC-MALLS)

To try and determine a more accurate estimate of the size of the polymer and obtain more information about the *Listeria* polymer structure, it was subjected to SEC-MALLS analysis. In this case, polymer that was not purified using the molecular weight cut off filters was used for the analysis which was performed as described by Morris *et al.* (2009) with the help of Dr Morris at the National Center for Macromolecular Hydrodynamics. This involved running the sample through two SEC columns with on-line MALLS

The eluent buffer was pumped into the columns and a 100 μ l volume of either the *Listeria* polymer or the PGA was injected in to the column. The absolute weight-average molar masses were calculated using the refractive index increment. Figure 7.7 shows the patterns produced by PGA and the *Listeria* polymer.

Even though the *Listeria* EPS material was not now thought to be PGA, this molecule was used for comparison as it has been previously well studied and known to have a molecular weight of approximately 1595 kDa and therefore results obtained in this analysis could be compared with previously reported data. The *Listeria* polymer was smaller in size than the PGA and had an average apparent molecular weight of \sim 4 kDa while the PGA size was determined to be 207 kDa against the expected value of 1595 kDa.

Figure 7.7 SEC-MALLS spectra of PGA and *Listeria* polymer



SEC-MALLS results obtained from analysis of *Listeria* polymer (—) and PGA (—). Two SEC columns (TSK G6000PW and TSK G4000PW protected by similarly packed guard column; Tosoh Bioscience, Japan) were used to carry out analytical fractionation with on-line MALLS. The eluent (0.2 M acetate buffer pH 4.3) was pumped at 0.8 ml min^{-1} and a $100 \mu\text{l}$ volume of both the *Listeria* polymer and PGA were injected. The absolute weight-average molar masses were calculated with ASTRA[®] software (Version 5.1.9.1, Wyatt Technology, and U.S.A.)

Morikawa *et al.* (2006) purified PGA from *Bacillus subtilis* B1, and measured the molecular weight by high performance silica gel thin layer chromatography and found the molecular weight to be over 1000 kDa. However, in their review Ashiuchi and Misono (2002) reported that the molecular weight of PGA could range from 10 to over 1000 kDa depending on the strain of *Bacillus* from which the PGA is extracted. They also highlighted the fact that in (*Bacillus subtilis* and *licheniformis*) during production of PGA, a PGA depolymerase accumulates which degrades the PGA and cause different range of molecular weight to occur.

It is possible that the *Listeria* polymer synthesis is also accompanied by a degrading enzyme that naturally degrades the polymer when it is no longer required by the cells. If this is true, the particular batch used for this experiment may have undergone some degradation. Alternatively the method was not the most suitable for estimating the molecular weight of the *Listeria* polymer.

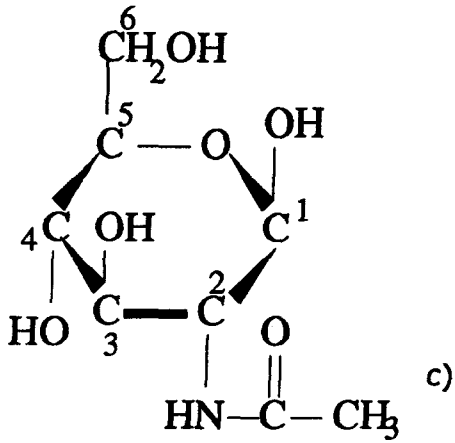
Gidh *et al.* (2006) used SEC-MALLS to monitor and characterize lignin and found that the accuracy is dependent on the ability of the UV and RI (refractive index) detectors to detect the material. While the lignin aggregation was detected by MALLS, it was not observed on the UV and RI detectors. In this study the signal obtained from the UV and RI detector for the *Listeria* EPS was poor compared to that of PGA which had distinct signals.

Irrespective of the accuracy of this method used to determine the sizes of these polymers one major difference noted during this analysis was that there was difference in the elution time for the two polymers tested. The *Listeria* polymer took over 25 min to elute whereas the PGA samples required on 20 min. This provides another confirmation that the *Listeria* polymer is not PGA.

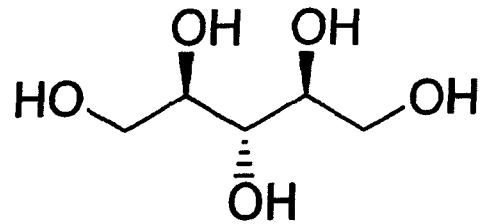
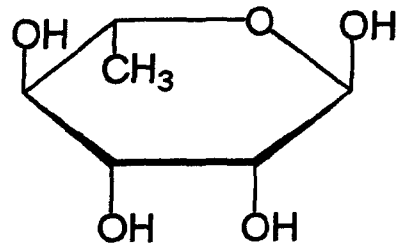
Using alkali extraction Kamisango *et al.* (1983) isolated an immunologically active teichoic acid component from the cell wall of *L. monocytogenes* by which accounted for about 20% of the weight of cell wall. Although it was not proposed that the compound was shed from the cells, it contained *N*-acetyl glucosamine, rhamnose, ribitol, and phosphorus. The molecular weight of this material was about 120,000 Daltons when analyzed by gel filtration. While rhamnose, ribitol and phosphorous would fit the chemical profile of the purified material, clearly glucosamine would not as it contains an N atom and would have been detected in the sugar analysis (see Fig. 7.8).

Figure 7.8: Structures of glucosamine, rhamnose and ribitol

a)



b)



Structures of (a) glucosamine (b) rhamnose and (c) ribitol; images taken from commons.wikimedia.org

7.6 Nuclear magnetic resonance analysis of purified *Listeria* EPS

This was performed in an attempt to gain more information about the chemical nature of the *Listeria* polymer. Both conventional high resolution liquid and solid state NMR were carried out on the polymer to confirm the structure of the EPS. Both types of NMR were carried out since the alignment of molecules which affects the signal produced is different in solids and liquids.

The liquid NMR was carried out at the School of chemistry with the help of Dr Adrienne Davis while the solid state NMR was performed with the help of Dr Bill McNaughton of the Food Structure group. For liquid NMR, the ^{13}C NMR spectra were acquired on a Bruker Av 500 spectrometer and again PGA was used as a reference compound.

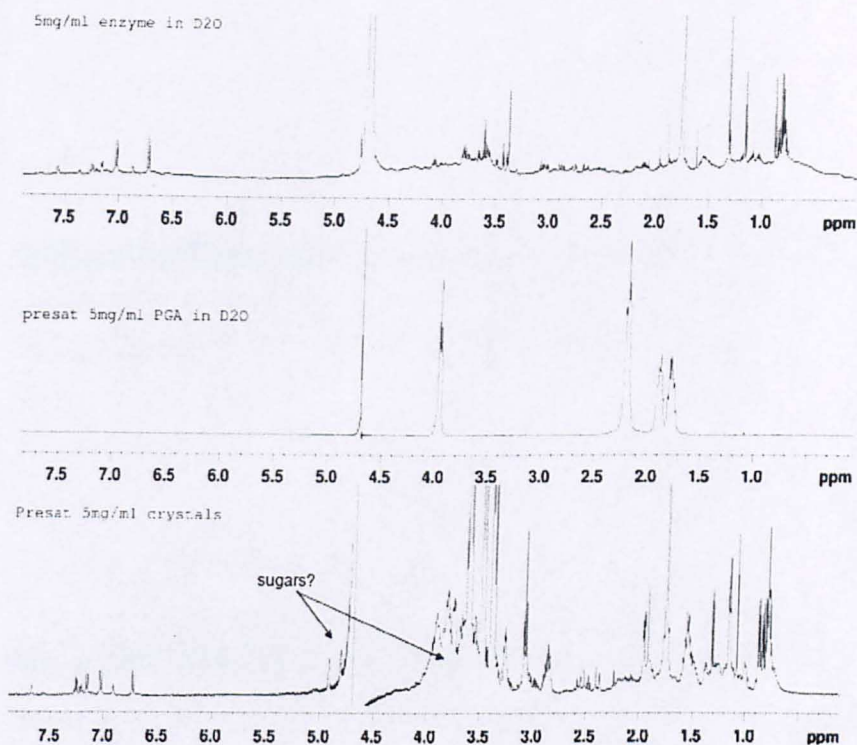
The enzyme pronase was used to remove associated cell proteins (section 2.22) and a sample of this enzyme was also analyzed to rule out the possibility of enzyme contamination of the signal. To perform the analysis 5 mg each of *Listeria* EPS, PGA and Pronase enzyme were dissolved in 1 ml of deuterium water (4%). The sample was placed in NMR tubes meter and the spectrum obtained was viewed on the visual display screen.

The proton NMR results again confirmed that the *Listeria* polymer was not the same as PGA since the spectra were completely different (Fig 7.9). The ^{13}C NMR analysis was performed at a higher EPS concentration (30mg/ml) also showed that the two compounds were not the same, and that unlike PGA the *Listeria* EPS seemed to contain abundant amounts of glycerol (Fig 7.10).

The identity of this peak corresponding to glycerol was confirmed by spiking the sample with commercial glycerol and increase in the corresponding peaks were seen. While the spectrum of PGA sample was different to that of the *Listeria* EPS, the pronase sample results did

share some similar regions. Some areas of the EPS were different and had low peaks which were close to peaks normally obtained for sugars but the peaks were not repeating enough to confirm them as sugar peaks.

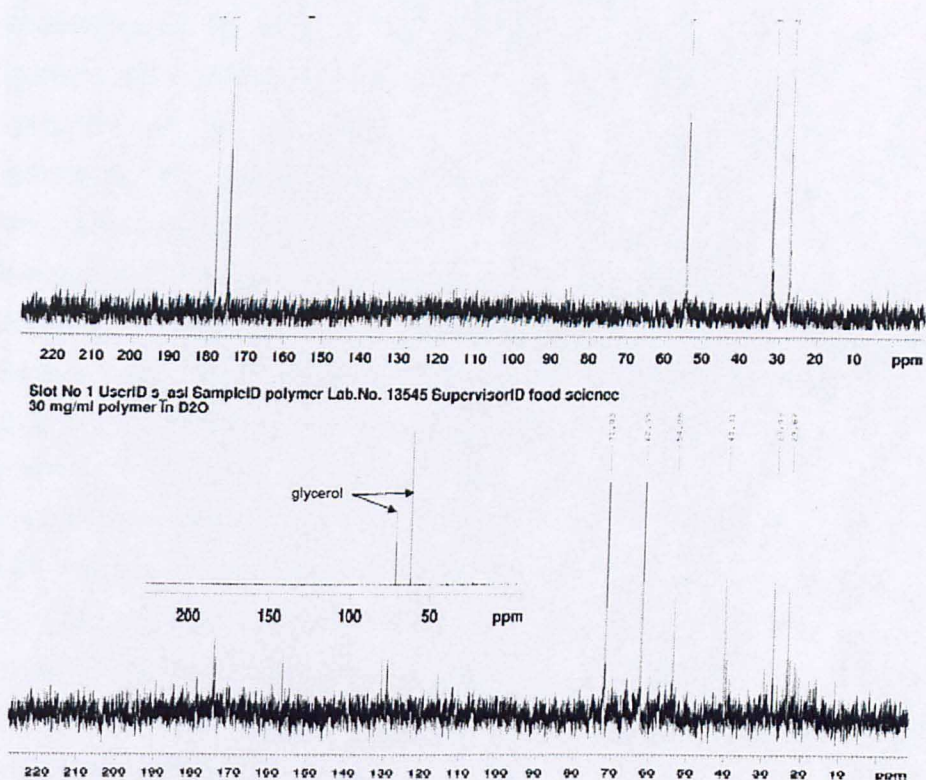
Figure 7.9 Proton (Liquid) NMR spectra



Spectra of 5 mg/ml PGA, pronase and *Listeria* EPS solutions detected by ^1H NMR after dissolving in deuterium water. The samples were placed in NMR tubes up to the 4 mm mark and placed inside the spinner of the spectrophotometer. The sample was then loaded into the magnet before acquiring and processing peaks by peak picking. Some peaks are suspected to be sugars but this result was not definitive due to high signal to noise ratio. Spectrum of enzyme used is shown on top and that of PGA middle while *Listeria* polymer spectrum is the bottom spectrum.

Figure 7.10

Carbon (Liquid) NMR of EPS compound



Comparison of 512 scan of 5mg *Listeria* EPS (top) and 14000 scan of 30mg *Listeria* EPS (below) dissolved in 4% deuterium water. Spectra were acquired with ^{13}C NMR by peak picking. Glycerol was visible in the 14000 scan, but was not seen in the 512 scan.

7.6.1 Cross polarization magic angle spinning (CPMAS) and solid state NMR of *Listeria* polymer

According to Stejskal & Memory (1994), attempts to obtain NMR spectra from solids showing clear chemical shifts, i.e. a series of well resolved peaks corresponding to nuclei in different chemical environments, are normally thwarted by a combination of factors. Also the existence of intense local fields from neighboring nuclei, results in broad spectral lines as a result of identical nuclei experiencing different magnetic fields and so resonating at different frequencies. Furthermore, this can be so marked as to result in the signal being very broad, merging with the background and so becoming invisible. Similarly the existence of chemical shift results in the resonance frequency of particular groups being different when oriented differently with respect to the magnetic field and so producing peak broadening. In addition, some solids can have very long relaxation times, resulting in very long times between successive scans being necessary in order for the signal to recover sufficiently to provide a reasonable intensity and thus acceptable signal to noise ratio.

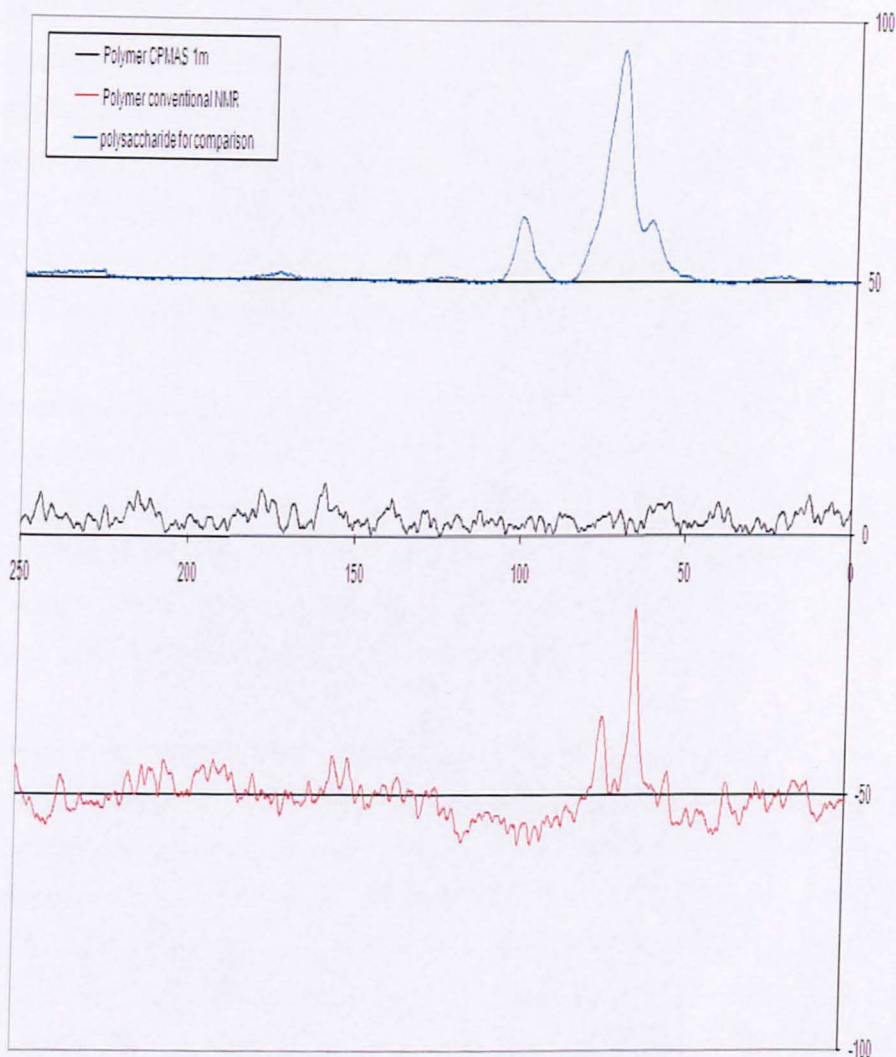
All these difficulties can be overcome to a degree, dependent on the sample, using the technique known as cross polarization magic angle spinning (CPMAS). High power decoupling overcomes the dipolar broadening whilst spinning at the magic angle and the cross polarization stage ameliorates the chemical shift and long solids problem respectively.

In this study the polysaccharide Xanthan was used as reference molecule. The dry Xanthan (Fig 7.11) sample gives a spectrum in CPMAS, showing the anomeric carbon resonances at ~ 100 ppm. The solid state CPMAS does not give any signal for the *Listeria* polymer. However, when run with single pulse excitation, the signal now resembles the conventional high resolutions NMR indicating the presence of glycerol with showing a high degree of mobility in the

Listeria polymer sample. Additionally, a lack of signal at 100ppm indicates the lack of sugar in the sample.

This again confirms the earlier findings that no complex sugars were present in the sample (section 7.3) and negates the possible identification of a signal indicative of sugar identified during the liquid NMR analysis.

Figure 7.11 CPMAS and Solid NMR analyses



Comparison of Solid state NMR spectra of Polysaccharide Xanthan (—) and the SPMAS (—) and CPMAS (—) spectrum of *Listeria* EPS. The Proton 90° pulse length was approximately $6.5 \mu\text{sec}$. Values of contact time of 1 and 5 msec were selected. Field strength of the proton and spin locking fields was approximately 40KHz. Samples were packed into 7mm rotors and spun at speeds of 3.75KHz. Recording of 8-16K data points were recorded with a time between points (dwell time) of $10 \mu\text{seconds}$. 20 Hz of Lorentzian line broadening was then applied. The data set was Fourier transformed and phased with 0 and 1st order corrections.

7.7 ATR-FTIR spectroscopy of purified *Listeria* EPS

The aim of this analysis was to provide more evidence that the *Listeria* polymer was not either protein or PGA-based, and to confirm earlier findings that indicated that the polymer was neither PGA nor a polysaccharide. The study was carried out with the help of Dr Mita Lad from the Food Structure group. FTIR spectra of dry powdered samples of the *Listeria* EPS, Pectin, BSA, PGA, Pronase and DNA were used. Pectin was used as a representative carbohydrate-based polymer while BSA was used as a representative protein.

Harmsen *et al.* (2010) reported that the dominant extracellular component of the *Listeria* EPS was extracellular DNA which originated from the chromosome. To investigate whether the *Listeria* polymer purified here contained DNA, *Listeria* chromosomal DNA was extracted (section 2.8) from the same strain used to prepare the *Listeria* EPS (Lm 10403S) and then subjected to FTIR analysis.

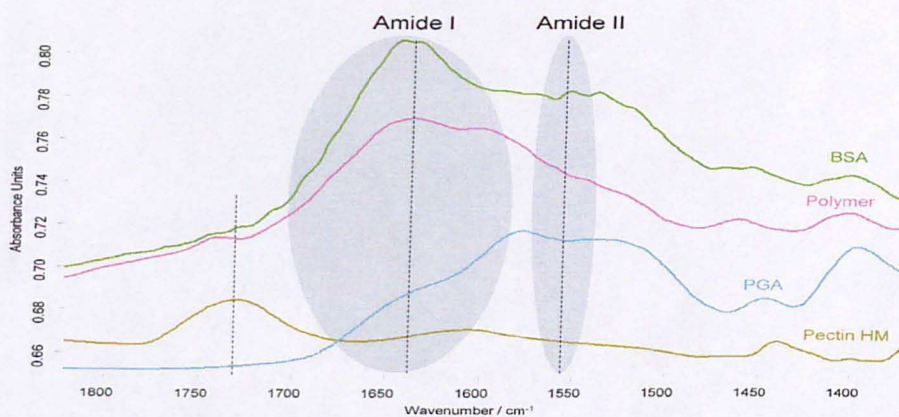
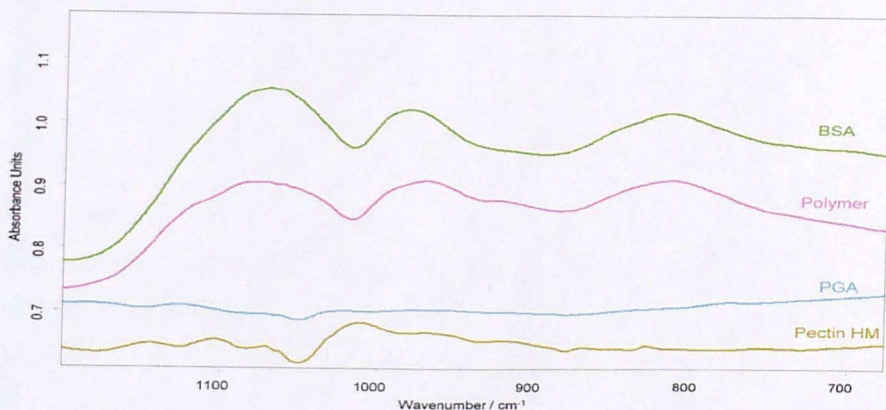
Results indicated once again that the *Listeria* polymer is neither protein, PGA or a polysaccharide. However there were some similarities in the amide regions (internal residual protein bonds) found between the spectrum of *Listeria* EPS and BSA (Fig 7.12) indicating that they could contain some common bond structures. In contrast when the Pronase enzyme spectra (Fig 7.13) was compared to the *Listeria* EPS it was found that the *Listeria* polymer was completely different to the pronase enzyme confirming that the enzyme had a different spectrum to the polymer and was not contributing to the spectra of the *Listeria* EPS recorded.

Even when the polymer's spectrum was deconvoluted - a process that reduces the width of absorption bands to resolve overlapping bands yielding a spectrum with narrower bands that allows closely spaced features to be distinguished - there was a clear difference between the spectra of the enzyme and the *Listeria* polymer.

The spectra of the DNA was compared to the spectra of the *Listeria* EPS and it was again found to be quite different (Fig. 7.14). This suggests that the dominant compound in the *Listeria* EPS is not DNA.

Figure 7.12 ATR-FTIR spectra of the polymer compared with proteins and carbohydrates

a)

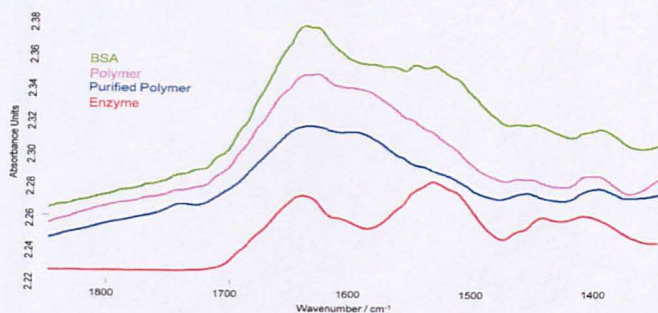


b)

ATR-FTIR spectra recorded for extracted polymer, BSA, PGA and pectin (a) and the fingerprint region of extracted polymer, BSA, PGA and pectin. A small amount of the powdered sample material was placed onto the diamond and contact with the diamond achieved by pressing the sample down using the anvil. Data were collected as interferograms at 4 cm⁻¹ resolution, where 128 interferograms were collected and co-added. Spectra were obtained by ratioing against a background spectrum of air. The results were recorded using the diamond golden gate accessory (Specac). For clarity, spectra were presented offset.

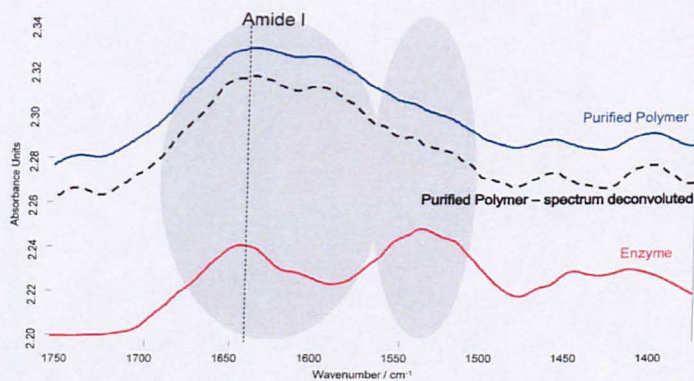
Figure 7.13 ATR-FTIR spectra of enzyme (pronase) used

A)



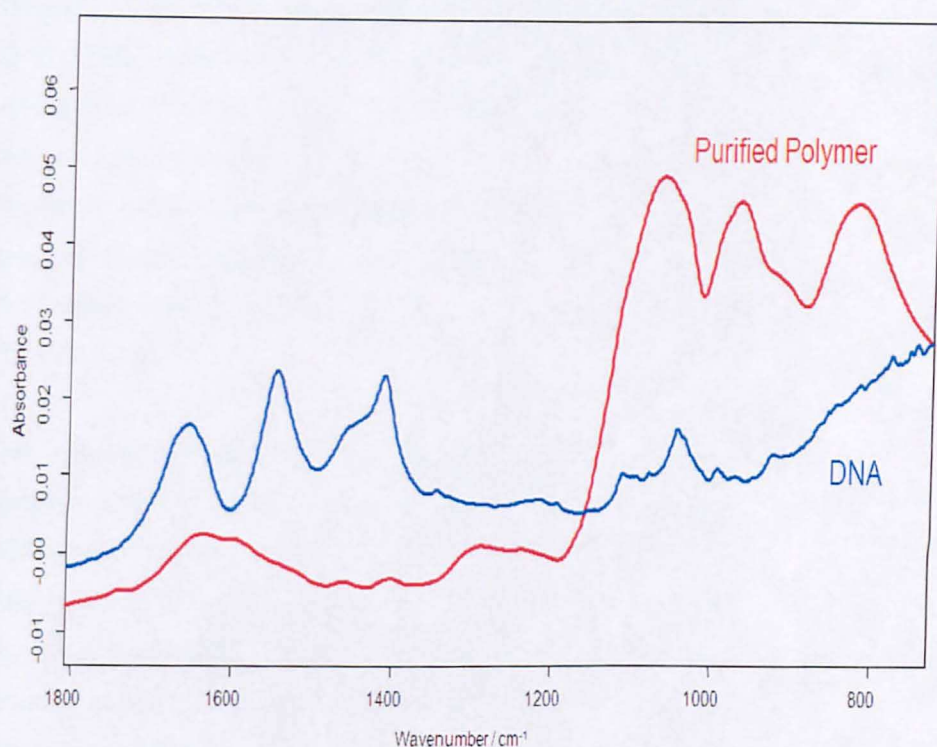
NB: Polymer = Polymer untreated with Pronase; Purified Polymer = Polymer treated with pronase.

B)



FTIR spectroscopy of the dry powder or crystals of each component (a) the enzyme (pronase) was used to compare because the NMR analysis showed the enzyme to have some regions in its spectra similar to the EPS extract. Fourier self-deconvoluted spectrum (b) shows that upon deconvolution the extracted polymer spectrum does not reveal the peak seen in the enzyme spectrum

Figure 7.14 ATR-FTIR spectra of DNA from Lm 10403S



The FTIR spectrum of purified *Listeria* EPS compared with that of DNA showing different spectra. The spectra were recorded using a Bruker detector (Tensor 27, Germany). FTIR spectra were recorded using a Bruker (Tensor 27, Germany) fitted with a deuterated triglyceride sulphate (DTGS) detector. FTIR was connected to an air dryer (Balston, US) to purge the instrument of water vapour and carbon dioxide.

7.8 X-ray diffraction

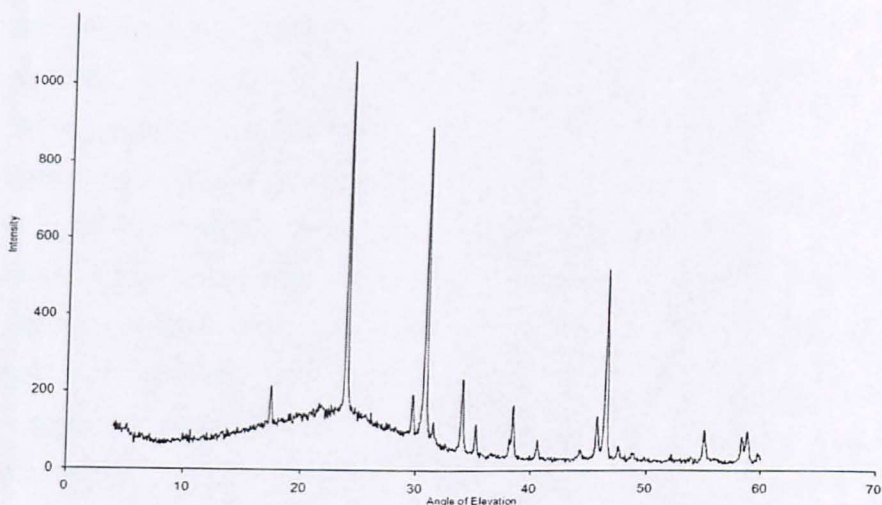
The methods described to date establish that the *Listeria* EPS is not a protein, is not PGA and does not contain a significant amount of sugar. Only NMR analysis showed a specific compound present in the material which was identified as glycerol. In a further attempt to determine the actual compound, or compounds that formed the *Listeria* polymer, X-ray diffraction was performed on the *Listeria* polymer and again PGA was used as a comparative molecule. Again the study was performed in liaison with the Food Structure group with the help of Dr Bill McNaughton.

The results obtained are shown in Figure 7.15 and indicate that the *Listeria* polymer had amorphous and structured regions while that of PGA had only amorphous regions. The structured region in the *Listeria* polymer was identified as Archerite, a Potassium Dihydrogen Phosphate (KH_2PO_4). As this compound was a component of the D10 media (section 2.x) it is possible that this was a contaminant that had been concentrated during the extraction process.

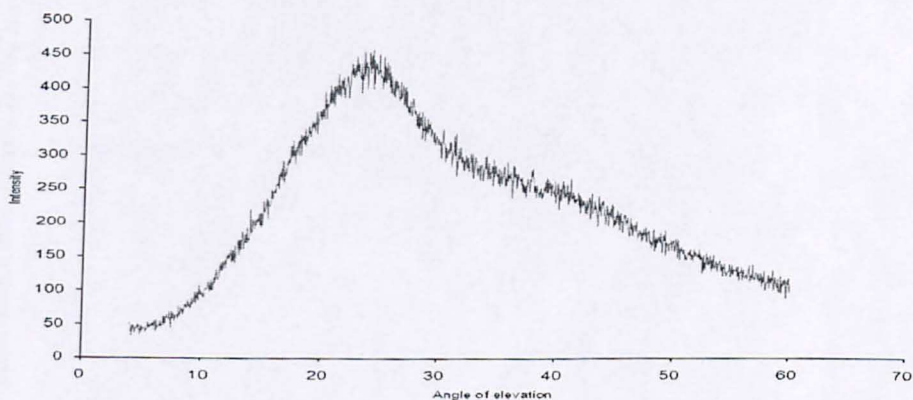
The amorphous region was large and occurred between about 12 and 36 degrees which indicate the presence of poorly crystalline material. This may be the polymer but it was not possible to identify what compound it was composed of with XRD as it was not crystalline enough. The PGA was not crystalline enough to detect any peaks, and the fact that this material when dried has a different form to the *Listeria* polymer provides another confirmation that it is not PGA.

Figure 7.15 X-ray diffraction of *Listeria* EPS and PGA

a)



b)



X-ray diffractograms of *Listeria* EPS (panel a) and PGA (panel b). X-ray diffractograms were recorded on powdered samples for values 2θ between 4 and 38° at 0.1° intervals with a scanning time of 6 s per interval, using a Bruker D5005 (Bruker AXS, UK) diffractometer at 20°C . Phase analysis and identification of crystalline forms were achieved using Bruker Diffrac^{plus} and eva software coupled to X-ray identification databases. The *Listeria* polymer and the PGA were found to be different.

7.9 Conclusions

From the work presented in this Chapter we now have some knowledge of the characteristics of the *Listeria* polymer. For instance the polymer can rapidly absorb water and can retain water for long periods. This property may contribute to the desiccation tolerance of these organisms when embedded in a biofilm matrix. Chemically, the three most abundant elements are carbon, oxygen and phosphorus, suggesting that it may be a carbohydrate. However the polymer was found to have very low sugar content but did contain large amounts of glycerol. Given the change in hydrophobicity of cells producing this polymer, and the presence of glycerol and phosphate, it could be suggested that the EPS is amphiphatic and may contain glycerol phosphate.

Further chemical analysis showed that it only contained amino acids in small amounts. This latter finding was the first clear evidence that the polymer was not PGA, or a polymer based on another amino acid, since no dominant amino acid was seen in contrast to PGA which clearly had glutamic acid as the dominant amino acid.

CHAPTER 8: GENERAL DISCUSSION

8.1 Discussion

From the experiments performed in Chapter 3 and 4, we came to the understanding that *Listeria* behaves differently when grown in minimal media. This fits with findings of other workers who have shown that *Listeria* can change its properties when the cells are exposed to a challenging environment. It has been mostly attributed to genetic and physico-chemical changes.

Olsen *et al.* (2009) found that when *Listeria* cells were exposed to acidic and salt stress, the virulence genes were up-regulated. Also Ryan *et al.* (2010) studied the effect of a 5 gene stress survival islet and found that they contribute to *Listeria* survival in sub optimal condition. When they created a mutant that lacked the islet, they found that it was impaired in growth at low pH and high salt concentration.

Other physico-chemical parameters that have been mentioned that causes changes in *Listeria* behaviour include temperature (Zhang *et al.*, 2010), hydrostatic pressure (Van Boeijen *et al.*, 2010) and initial redox potential (Ignatova *et al.*, 2010). But now we are seeing changes in the surface of the cells brought about by nutrient limitation and cells grown in minimal media had the most change in surface properties. Nutrient limitation has been shown to make *Listeria* to become more resistant to stresses. O'Bryan *et al.* (2009) found that *Listeria* cells starved for 14 days were more resistant to Nisin than cells grown under normal cell cultivation.

The next line of research may be to identify exactly, the physico-chemical condition that encourage the changes in surface properties and this would ultimately lead to the identification of the genes that

encourage *Listeria* to change its surface properties in a nutrient starved environment.

In their review, Van Houdt and Michiels (2010) found that changes in the surface properties have been attributed to several factors which include temperature, pH and quorum sensing. Furthermore, in an unpublished work by members of this research group (H-T. Wong, personal communication) it was shown that effect of minimal growth media on the adhesion of *L. monocytogenes* may be mediated by AI-2 autoinducer. In the study using 3 sample strains (Lm 10403S, Lm 23074 and Lm EGD-e), a great reduction was seen in AI-2 production in the minimal media MCDB 202 compared to that in the rich media BHI. This indicated that there is a reduction in AI-2 production when *L. monocytogenes* strains are grown in a nutrient- limited media. Prior to that work Sela *et al.* (2006) showed that the ability to form biofilms in *Listeria* is affected by the synthesis of a functional autoinducer 2 (AI-2)-like signal, and that an intact *luxS* gene is associated with inhibition of attachment and biofilm formation. Hence the growth media used may be affecting global cell signalling which results in the changes in surface properties seen in this study.

8.2 Definitive findings

Cells grown in minimal media were more hydrophobic than those grown in rich media and the hydrophobicity was enhanced when duck juice was added to the minimal media. The variation seen in the Chapter 4 experiments may be due to the experimental method and various factors which affect hydrophobicity. Many workers have tried to interpret the factors that affect hydrophobicity and it has been pointed out that hydrophobicity assays essentially probe interplay of all physico-chemical and structural factors involved in microbial adhesion rather than one single factor i.e. the cell surface hydrophobicity (Van der Mei *et al.* 1995). Some of these factors mentioned previously

include Van der Waal and electrostatic forces (Bellon-Fontaine *et al.*, 1996) and cell surface charge and electron and acceptor properties of the cell (Briandet *et al.*, 1999).

Takahashi *et al.* (2010) found that cellular hydrophobicity is an important property involved in the initial adherence to PVC surfaces while Chae *et al.* (2006) suggested that initial adherence to glass surfaces is dependent on electrostatic force but not hydrophobicity while Ukuku and Fett (2002) reported that initial adherence is on fruits is correlated with hydrophobicity of *L. monocytogenes*.

A way to overcome this uncertainty may be to use the reference guide to microbial cell surface hydrophobicity based on measurement of contact angles proposed by Van der Mei *et al.* (1998). They advised that using contact angles to measure hydrophobicity ensures that no generalizations concerning the physico-chemical surface properties of microorganisms maybe made. Therefore, it would be beneficial if the hydrophobicity in these four different media is reassessed based on contact angle measurements to eliminate things like effect of buffer used which appeared to be a factor when the hydrophobicity assays were carried out in Chapter 3 and 4.

Another finding is that we have established that EPS which is regarded as the house of biofilms can be made by planktonic cells. Most description of biofilm formation shows that the planktonic cells need to attach to each other before EPS and biofilms are made (Davey and O'Toole, 2000). A novel finding in this study was the establishment of the fact that for the first time that *Listeria* was able to make a capsular polymer especially when grown in minimal media. Capsular materials are generally polysaccharide in nature and since it is generally known that *Listeria* do not make polysaccharides, we had to confirm initial observations with a second capsule stain (the Giemsa stain) which also gave a positive result.

The nearest finding to date regarding the ability of *Listeria* to make a surface carbohydrate was when Boruki *et al.* (2003) used Ruthenium dye, a carbohydrate stain to stain *Listeria* biofilm cells. They concluded that the presence of the pink stain between cells was consistent with an exopolysaccharide matrix. They abbreviated exopolysaccharide as EPS but for our study, we regarded EPS as exopolymeric substances and indeed chemical analysis of the polymer did not find any evidence of a sugar component indicated that the polymer may not be exopolysaccharide in nature. It is possible that Boruki *et al.* (2003) detected simple sugars which were also detected in our material at low level after the sugar analysis was performed. Again it is possible they picked up sugar present in eDNA. Harmsen *et al.* (2010) have shown that *Listeria* EPS contains eDNA. Other workers have mostly used the ruthenium stain to stain solid biofilms (Chae *et al.*, 2006) and the general consensus is that there must be a genetic trigger that allows *Listeria* to make an exopolymeric matrix. Further studies to identify the genes responsible may help to unravel the mystery.

The interest in the *L. monocytogenes* cellular envelope have increased over the years and many description of the suspected peptidoglycan and associated polymers were made over 20 years ago (Bierne and Cossart, 2007). So when we undertook further analysis to determine the exact nature of the polymer, we got clear evidence that the polymer was not PGA or protein but the high carbon content showed that the material was organic. We then concluded that the material may be phospholipids since high amounts of glycerol and phosphorous were detected.

The challenge of this identification process was that several methods were used and there was no *Listeria* EPS standard. However, the X-

ray diffraction analysis indicated that an unidentified compound may be the actual polymer. It may be necessary to rule out fatty acids and lipids by assaying for fatty acids.

The enhancement of growth and hydrophobicity by duck juice supports that proposal that the polymer may be fatty acids. Duck meat is fatty in nature and recent findings gives this more credence. Using a bioinformatics approach Webb *et al.* (2009) identified two-enzyme systems for glycolipid and polyglycerolphosphate lipoteichoic acid (LTA) synthesis in *L. monocytogenes*. They identified *L. monocytogenes* genes predicted to be involved in glycolipid (*Imo2555* and *Imo2554*) and LTA backbone (*Imo0644* and *Imo0927*) synthesis. LTA and glycolipid analysis of wild-type and mutant strains confirmed the function of *Lmo2555* and *Lmo2554* as glycosyltransferases required for the formation of *Listeria* glycolipids. It would be interesting to see whether these genes are up regulated or down regulated in cells grown in the 4 different media used in this study.

Also Giannotti *et al.* (2008) reported that when adhered and free floating cells of two *Listeria* strains were separately exposed to acid stress, an increase of the individual and total branched fatty acids was observed particularly in the floating cells of the two strains. The acid stress gave rise in the adhered cells of strain endowed with the lower biofilm forming ability to a relevant intracellular accumulation of straight medium chain fatty acids. Straight long chain and medium chain free fatty acids were released in the culture supernatants particularly by the strain endowed with a high biofilm forming ability.

8.3 What is the biological role of *Listeria* Polymer?

So would the polymer increase biofilm formation? The answer for now is likely to be no. This is because our findings were made with planktonic cells in liquid culture and biofilm is normally associated with solid surfaces. In fact, most recent work on biofilms has been carried

out on solid surfaces (Luksiene *et al.* 2010, Vaid *et al.* 2010; and Olmez *et al.* 2010). Although the hydrophobicity tests in this thesis were not performed on solid surface, we can say that the hydrophobicity in this study is affected by cells grown in minimal media.

But the polymer may likely help the *Listeria* cells survive long desiccation periods since it was shown that it could retain water overnight. Vogel *et al.* (2010) studied the survival of *L. monocytogenes* studied during long term desiccation in a fish slaughter house and showed that the ability to survive for months during desiccated conditions may be a factor explaining the ability of *L. monocytogenes* to persist in food processing environments.

Also Porsby *et al.* (2008) looked at the influence of processing steps in cold-smoked salmon production on survival and growth of persistent and presumed non-persistent *L. monocytogenes*. They found that the processing steps involved in cold-smoking of salmon are bactericidal and reduce, but will not eliminate, *L. monocytogenes*. Furthermore, a persistent strain was no less sensitive to the processing steps than a clinical strain or strain EGD-e. More information can be obtained if the purified polymer is subjected to factory conditions to verify that it can withstand long desiccation periods.

The polymer may also provide protection for *Listeria* cells and offer resistance to sanitation chemicals and make the cells less easily removed. Biofilms on solid surface have been shown to be more resistant than planktonic cells. In fact most resistant studies have been carried out on solid surfaces (Belessi *et al.*, 2010) It is now important to include planktonic cells grown in different media in such studies as little work has been carried out in that area.

More studies with this polymer are needed to be carried out in the factories so that we can develop sanitation procedures and reagents that can break down the *Listeria* EPS. If this is carried out, persistence and outbreak of Listeriosis would be reduced.

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