Studies on the adhesion of *Listeria monocytogenes* to leaf and model surfaces

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Abstract

Adhesion of *Listeria monocytogenes* to vegetable and food contact surfaces is important in the contamination of salad products, potentially leading to food-borne listeriosis. This study investigated the factors affecting the adhesion of *L. monocytogenes* to leaf and model surfaces with the ultimate aim of developing strategies to inhibit adhesion or detach adhering cells.

Negative staining of *L. monocytogenes* cells showed they did not produce adhesive structures such as fimbriae, pili or fibrils under eight different growth regimes.

Three adhesion assays were developed to assess adhesion to artificially-inoculated surfaces under a range of physico-chemical conditions. Adhering and planktonic cell populations on leaf surfaces were monitored using microbiological counts. Scanning electron microscopy (SEM) was used to quantify total adhered cells and assess their spatial distribution on leaf and model surfaces. Photometric analysis, using crystal-violet staining of fixed cells, was used as a rapid *in vitro* technique to assess adhesion to model dental wax surfaces.

Adhesion to Iceberg lettuce, Dutch White cabbage and dental wax surfaces reached maximum levels within 24 h of incubation and was dependent on the ionic composition of the cell-suspending medium. At 24 h adhesion to lettuce and cabbage increased significantly from 0.05 and 0.02% (deionised water) to 0.3 and 4.5% (1/4 strength Ringer's solution) respectively. Maximum adhesion to cabbage tissue (6.6%) was recorded for cells suspended in 40 mM $MgCl_2.6H_2O$. Adhesion levels to dental wax ranged from 1.0×10^4 to 4.0×10^7 cells cm^{-2} dependent on electrolyte type and concentration; percentage adhesion increasing from 0.001% (deionized water), 0.6% (40 mM NaCl and KCl), 2.7% (1/4 strength Ringer's solution) to maximum levels of 4.9% for cells suspended in 40 mM $MgCl_2.6H_2O$. Parallel analysis using bacterial adhesion to hydrocarbons (BATH) also demonstrated an increased cell-surface hydrophobicity in the presence of electrolytes. Electrolytes may increase adhesion through reduced electrostatic repulsion, as described by the DLVO theory, and/or increased hydrophobic bonding, each effect is discussed. Monovalent cations did not increase adhesion to the maximum levels observed for divalent cations (independent of electrolyte concentration) suggesting that divalent cations may also enhance adhesion through bridging interactions.

Substratum hydrophobicity was quantified by the measurement of mean advancing ($M\Theta a$) and receding water contact angles using the Wilhelmy plate technique. Cabbage tissue was hydrophobic ($M\Theta a = 98.3°$) and lettuce relatively hydrophilic ($M\Theta a = 65.7°$). Advancing contact angles for model surfaces ranged from 25.5° to 135.7° and a trend of increasing adhesion was observed with increasing hydrophobicity in this range. Adhesion to hydrophobic dental wax was reduced by at least 60% with the addition of 3% DMSO or 50 ppm of Brij 56 or CHAPS to the cell-suspending medium. Results suggest that hydrophobic bonding increases adhesion of *L. monocytogenes* to hydrophobic surfaces. Positive correlation between increasing substratum roughness, assessed by Ra measurement, and adhesion to model surfaces was also demonstrated. Spatial distribution of adhering cells on leaf and model surfaces is discussed in relation to the substratum chemical and physical heterogeneity.

Ultraviolet irradiated cells adhered to dental wax but did not form micro-colonies indicating that initial adhesion may occur through physico-chemical interactions but subsequent colonization is dependent on active mechanisms and possibly positive cooperativity between adhering and planktonic cells. Increased adhesion to dental wax was also demonstrated during late bacterial growth phase and following cell deposition.

The biological surfactants Brij 56 and SDS were more effective than distilled water in removing adherent cells from dental wax surfaces. The effective washing concentrations of these surfactants were > 100 ppm but ≤ 1000 ppm.

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university of other institute of learning.

Mark David Brown

1996

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Dedication

I would like to dedicate this thesis to my parents. During my studies they have encouraged me to work to the best of my abilities and supported my efforts with love and understanding. For this I am eternally grateful.

List of Abbreviations

Item	Abbreviation
Arithmetical mean roughness	Ra
Bacterial Adhesion to Hydrocarbon Test	BATH
Benzethonium chloride	BC
Brain Heart Infusion broth	BHI
Cabbage wax-coated	CBW
Cetyldimethylammonium bromide	CB
Chloroform washed	CW
Colony forming units	cfu
Critical control point	CCP
Dimethyl sulfoxide	DMSO
Dynamic contact angle	DCA
Flame oxidised	FO
Listeria selective agar	LSA
Logarithm to base 10	$\log_{10}$
Mean advancing water contact angle	MΘa
Mean receding water contact angle	MΘr
Mean levelling depth	Rpm
Mean peak to valley height	Rz
Minimum detection level	M.D.L
Not determined	ND
Not treated	NT
Optical density at 420 nm	$O.D._{420}$
Optical density at 570 nm	$O.D._{570}$
Plasma-glow discharged	PGD
Scanning electron microscope	SEM
Standard deviation	S.D.
Standard error of the mean	S.E.
Surface-active agent	SAA
Thermodynamic water contact angle hysteresis	▲Θ
Tryptone Soy agar	TSA
Tryptone Soy broth	TSB
Ultraviolet	U.V.
Volume to volume	v/v
Weight to volume	w/v
Zero depth of immersion	ZDOI

Chapter One

Introduction

1.1 History of listeriosis.

Gray & Killinger (1966) published a classic review of *Listeria monocytogenes* (Murray, Webb & Swan, 1926); Pirie (1927), and listeric infections in humans and other animals. Since then, *L. monocytogenes* has been implicated as the causitive agent in several food-borne outbreaks of listeriosis throughout North America and Europe. As a result of such outbreaks during the 1980s, interest in the organism grew rapidly among food manufacturers, government bodies and the media. This resulted in a concomitant increase in the published literature (ANON, 1988a; El-Kest & Marth, 1988a; Gellin & Broome, 1989; Farber & Peterkin, 1991) and a rise in public interest of the disease.

According to Gray & Killinger (1966), the first recorded case of listeriosis was made in France in 1891 by Hayem. Hayem observed Gram-positive rods in sections from a patient who had died from a disease that, in retrospect, almost certainly was a listeric infection. Hulphers (1911) appears to have been the first person to isolate *L. monocytogenes* from animal tissue. He isolated an organism which he called *Bacillus hepatis* from the necrotic foci in the liver of a rabbit. His description of the organism corresponds very closely to that of *L. monocytogenes*.

The first detailed description of *L. monocytogenes* was published by Murray *et al.* (1926). They named the bacterium *Bacterium monocytogenes* because a large number of monocytes were found in the peripheral blood of infected laboratory rabbits and guinea pigs. Pirie (1927) isolated an identical bacterium from the liver of several gerbils. In the same year he named his isolate *Listerella hepatolytica* in honour of Lord Lister but in 1940 he re-named it *L. monocytogenes* (Pirie, 1940).

The first confirmed report of listeric infection in man was made by Nyfeldt (1929). He isolated the bacterium from three patients with an infectious-like disease. Burn (1936) described *L. monocytogenes* as a cause of perinatal infections in humans.

It is now known that the bacterium can be isolated from a very wide range of hosts including at least 37 warm-blooded animal species, in addition to arthropods, fish, insect larvae, frogs and snails, and has a global distribution (Lovett & Twedt, 1988).

1.2 The genus *Listeria.*

1.2.1 Taxonomy of the genus *Listeria.*

Both the intra- and intergeneric taxonomy of bacteria of the genus *Listeria* have been problematic for a number of years (Farber & Peterkin, 1991). The latest edition of Bergey's Manual of Systematic Bacteriology lists *Listeria* in a section entitled Regular, Non-sporing, Gram-Positive Rods (Seeliger & Jones, 1986). Until 1961 *L. monocytogenes* was the only recognised species in the genus. To date, however, the genus comprises 7 species. These are: *L. monocytogenes.*, *L. innocua*, *L. ivanovii*, *L. welshimeri*, *L. seeligeri*, *L. grayi* and *L. murrayi* (Farber & Peterkin, 1991).

On the basis of numerical taxonomic, serological and DNA/DNA hybridisation studies, a very close relationship has been shown between *L. grayi* and *L. murrayi* which is distinct from the rest of the genus (McLauchlin, 1987). Stuart & Welshimer (1974) proposed the creation of a new genus *Murraya* which contains *Murraya grayi* subsp. *grayi* (*L. grayi*) and *Murraya grayi* subsp. *murrayi* (*L. murrayi*). This designation has not become widely used, however, even though the genus is now generally considered to consist of only 5 true species.

1.2.2 Systems of typing and subtyping *L. monocytogenes.*

L. monocytogenes is subdivided into serotypes on the basis of somatic (O) and flagellar (H) antigens. *L. monocytogenes* may be sub-divided into four serogroups (1/2, 3, 4 & 7) and thirteen serovars 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, & 7 (Farber & Peterkin, 1991). Because the vast majority of cases of human disease are caused by only three serotypes (1/2a, 1/2b, and 4b), serotyping is only minimally helpful in epidemiological investigations (Schuchat, Swaminathan & Broome, 1991). Various subtyping methods have been applied to collections of *L. monocytogenes* isolates, including, phage typing (McLauchlin, Audurier & Taylor, 1986), multi-locus

enzyme electrophoresis (Salander *et al.*, 1986), restriction fragment length polymorphism (RFLP) (Norcera *et al.*, 1989), ribosomal DNA fingerprinting (ribotyping) (Swaminathan *et al.*, 1988) and random amplification of polymorphic DNA (RAPD) (Wagner, Maderner & Brandl, 1996).

Phage typing has been successfully applied to epidemiological investigations (Fleming *et al.*, 1985; Linnan *et al.*, 1988), however, quite a high proportion of strains cannot be typed and its use in epidemiological studies is therefore limited (McLauchlin *et al.*, 1986). Multi-locus enzyme electrophoresis has recently been proven to be an excellent method in epidemiological investigations (Bibb *et al.*, 1990; Nørrung & Skovgaard, 1993). This method differentiates organisms by variations in electrophoretic mobilities of a panel of consecutive enzymes. It is a particularly useful technique because it is capable of typing all *Listeria* isolates and provides information on the degree of genetic relatedness between strains.

1.2.3 Morphology and growth characteristics of *L. monocytogenes.*

L. monocytogenes is a facultatively anaerobic, non-sporeforming, non-capsulate bacterium (Gray & Killinger, 1966). The bacterium may appear as short rods measuring 0.5-1.0 μm in length, long rods 1.0-2.0 μm in length, or indeed as coccoid forms which are often seen in smears from infected tissue or broth cultures, but are rare in smears from colonies (El-Kest & Marth, 1988a). It can grow at temperatures between -0.4 and 45°C (Seeliger & Jones, 1986; Walker, Archer & Banks, 1990), and may proliferate at refrigerated temperatures making it of particular concern to food manufacturers. *L. monocytogenes* is oxidase negative and catalase positive and expresses an extracellular sulphydryl activated ß-haemolysin, lysterolysin O, which produces zones of clearing on blood agar. The organism possesses peritrichous flagella which, when grown at temperatures between 20 and 25°C, give the organism a characteristic tumbling motility. At 37°C the organism becomes non-motile due to a dramatic reduction in the production of flagellin (Peel, Donachie & Shaw, 1988).

L. monocytogenes is quite resistant to an alkaline pH, growing in liquid media at pH 9.6 (Doyle, 1988). Published reports indicate that *L. monocytogenes* will also

grow or survive at pH values as low as 5.2 to 4.3 (Ahmed & Marth, 1989; Farber *et al.*, 1989a; Cole, Jones & Holyoak, 1990; Conner, Scott & Bernard, 1990; McClure, Kelly & Roberts, 1991). Ahmed & Marth (1989) showed that of several acids (acetic, lactic, citric, and hydrochloric) used to lower the pH of Brain Heart Infusion broth, acetic acid was the most effective growth inhibitor. *L. monocytogenes* was capable of initiating growth at pH 4.3 to 5.2 at 30°C and pH 5.0 to 5.7 at 4°C depending on the acidulent used (Farber *et al.*, 1989a). Buchanan & Phillips (1990), recently developed a mathematical model describing the effects of temperature (5 to 37°C), pH (4.5 to 7.5), NaCl (5 to 45 g/litre), $NaNO_2$ (0 to 1,1000 μg/ml) and atmosphere (aerobic or anaerobic) on the growth kinetics of *L. monocytogenes* in Tryptone Phosphate broth.

Thus, the ability of this organism to survive for long periods of time under quite extreme environmental conditions of temperature, pH and salt concentration, makes it ideally suited to survival in the natural environment.

1.3 Natural reservoirs of *Listeria* species in the environment.

Since Gray (1964) suggested that 'the wide geographical distribution of listeric infection involving diverse species of animals suggests common reservoirs of *L. monocytogenes* shared by man and animals' many researchers have examined the natural environment for the presence of *Listeria*. Some of these environments in which *Listeria* has been isolated are described below.

1.3.1 Faeces.

Due to the enteric existence of the organism in the host (Section 1.4) and its very large host range, *L. monocytogenes* can frequently be isolated from faecal samples of wild animals and birds (Weis & Seeliger, 1975; Fenlon, 1985), dairy and beef cattle (Siragusa, Dickson & Daniels, 1993), sheep (Grønstøl, 1979), poultry (Buncic, 1991) and humans (Lamont & Postlethwaite, 1986).

Fenlon (1985) implicated bird faeces (particularly gulls) as a possible route of the organism into silage (see Section 1.6.1 for transmission of *Listeria* into animals through the consumption of contaminated silage). Skovgaard & Morgen (1988) and

Skovgaard & Norrung (1989) found that 1.7 and 52% of samples of fresh pig and fresh cattle faeces were positive for *L. monocytogenes*.

Several investigators have found *L. monocytogenes* in faecal specimens from healthy people (Kampelmacher & van Noorle Jansen, 1969; Lamont & Postlethwaite, 1986), and wide-ranging estimates for stool carriage of *L. monocytogenes* among healthy adults have been reported. As many as 13.3% of slaughterhouse workers were found to be carriers of *Listeria* (Kampelmacher & van Noorle Jansen, 1969). These facts are consistent with the suggestion that the gastrointestinal tract is the human reservoir for the organism.

1.3.2 Sewage sludge.

L. monocytogenes in concentrations of 10^3-10^4 cells ml^{-1} have been found in sewage plant effluent while sewage sludge has shown even higher cell concentrations (Lovett & Twedt, 1988). *L. monocytogenes* has been isolated in sewage sludge cake which is commonly used as an agricultural fertiliser in Iraq (Al-Ghazali & Al-Azawi, 1986, 1988). Field experiments have shown that crops grown in soils treated with this material became contaminated (Al-Ghazali & Al-Azawi, 1990). The use of raw sewage or sewage sludge or manure from farm animals as a fertilizer was, therefore, suggested to be a potential point of entry of *Listeria* into the food chain.

1.3.3 Water.

It is possible to isolate *L. monocytogenes* from surface rivers and streams (Watkins & Sleath, 1981), and van Renterghem *et al.* (1991) showed that 5% of ground water samples were contaminated with *L. monocytogenes*.

1.3.4 Soil.

Welshimer (1960) examined the survival of *Listeria* added to sterile soil samples over a period of 295 days. Moist fertile soils supported the survival of *Listeria* cells for longer periods than clay soils. Botzler, Cowan & Wetzler (1974) showed that *Listeria* is capable of growing in sterile soil samples under laboratory conditions, but the

organism did not appear to compete well with natural microflora in un-sterilised soils and eventually died out. Weis & Seeliger (1975) isolated *L. monocytogenes* from both cultivated and uncultivated soils. The organism was more often detected on the surface of soil specimens, particularly in fields which had lain fallow for several years and were overgrown with grass and small shrubs. Incidence was reduced at increasing depth of soil and with increasing cultivation.

Van Renterghem *et al.* (1991) artificially inoculated soil, liquid cattle and liquid pig manure with *L. monocytogenes*. Three to four weeks after inoculation *L. monocytogenes* was not detected in the pig manure. It was noted that this rapid die off may have been due to the presence of antimicrobial feed additives such as antibiotics and copper in pig manure. At six weeks *L. monocytogenes* was intermittently found in cattle manure and soil samples, but by eight weeks was not detected in any samples. In this study it was concluded that *L. monocytogenes* was not capable of surviving for long periods in soil or liquid manure and consequently these authors concluded that soil and manure were not natural reservoirs for *Listeria.*

In an extension to the above experiment, van Renterghem *et al.* (1991) cultivated radishes in soil fertilised with pig manure. Three months after inoculation they noted that 50% of unwashed radishes tested positive for *L. monocytogenes* while only 17% of the soil samples where the radishes had been previously cultivated contained the organism. It was, therefore, suggested that the plant-soil rhizosphere is the natural reservoir of *L. monocytogenes*.

1.3.5 Natural vegetation.

Seeliger (1961) commented on the resemblance of the biochemical and cultural characteristics of *L. monocytogenes* to some plant-soil inhabitants and speculated that 'there may well be a saprophytic life of *Listeria*'. Since then *L. monocytogenes* has been isolated from environmental sources of vegetation including dead and decaying corn and soy beans (Welshimer, 1968), wild grasses (Welshimer & Donker-Voet, 1971), from old, faded or mouldy plants and leaves of shrubs (Weis & Seeliger, 1975). Welshimer & Donker-Voet (1971) reported that the multiplication of *L. monocytogenes*

was increased by decay of organic plant material and speculated that *Listeria* may be isolated more frequently from portions of the plant nearer the ground.

1.3.6 Cultivated fruit and vegetables.

Recent surveys have indicated that *Listeria* species may be isolated from minimally processed fruit and vegetable samples at frequencies which range from 0 to 30% of samples (see Section 1.7.2 for further details of these surveys).

1.4 *Listeria* infection in humans.

Owing to the ubiquitous presence of *Listeria* in the environment, human exposure to *Listeria* is believed to be very frequent. However, despite this high frequency of exposure invasive listeriosis with syndromes such as meningoencephalitis and septicemia are rare (see Section 1.4.4 for description of these and additional disease syndromes). Factors which may influence whether invasive disease will occur include the virulence of the infecting organism (Sections 1.4.1 & 1.4.2) and susceptibility of the host (Section 1.4.3).

1.4.1 *Listeria* species causing disease.

Only the haemolytic species of *Listeria*, namely, *L. monocytogenes*, *L. seeligeri* and *L. ivanovii* are believed to be involved in pathogenicity in humans. Of these only *L. monocytogenes* is consistently pathogenic (Rocourt & Seeliger, 1985). *L. ivanovii* has been reported to be involved in human pathology in only three instances (Busch, 1971; Rocourt & Seeliger, 1985) and *L. seeligeri* has been reported only once to be the cause of meningitis in a non-compromised adult (Rocourt *et al.*, 1986). *L. monocytogenes* is now considered to be the major pathogen for both humans and animals (Chen, Wang & Levin, 1996).

All virulent strains of *L. monocytogenes* produce an extracellular sulphydryl activated ß-haemolysin, lysterolysin O. This haemolysin is believed to be a major virulence factor in the intracellular pathogenicity of the organism (Kuhn, Kathariou & Goebel, 1988; Czuprinski, 1994; Datta, 1994).

1.4.2 Intracellular pathogenicity of *Listeria* spp.

Epidemiological and experimental evidence documents the gastrointestinal tract as the natural route of listeric infection (Schlech *et al.*, 1983) (see Section 1.6 for epidemiological evidence of food-borne transmission). Intestinal epithelial cells act as a first barrier to the bacteria. Thus, the ability of the bacteria to adhere to and invade epithelial cells of intestinal villi is correlated with bacterial virulence (Meyer *et al.*, 1992). Gaillard *et al.* (1987) found that a non-haemolytic mutant of *L. monocytogenes* invaded a continuous gut epithelial cell line Caco-2 at the same rate as the haemolytic wild type. This demonstrates that haemolysin is not involved in invasion.

Not surprisingly, specific molecules on both the epithelial cell and the microbe are necessary for invasion to occur. Gaillard *et al.* (1991) identified an 80k-Da surface protein on the surface of *L. monocytogenes* cells named internalin that was essential for the bacteria to bind and invade epithelial cells *in vitro*. Non-invasive *L. innocua* isolates lacked this surface protein and an internalin negative mutant of *L. monocytogenes* was also unable to bind to or invade Caco-2 epithelial cells *in vitro* (Gaillard *et al.*, 1991). Mengaud *et al.* (1996) recently demonstrated that the cellular receptor for internalin is a glycoprotein called E-cadherin. E-cadherin plays a crucial role in the maintenance of intestinal epithelial structure and is expressed at the basolateral face of epithelial enterocytes. In many tissues more than one form of cadherin (including E-, N-, R-, M- and P-cadherin forms) can be found. It has therefore, been suggested that the nature and relative amount of cadherin expressed in one particular cell type could be the basis for species and cell tropism of *L. monocytogenes* (Mengaud *et al.*, 1996).

L. monocytogenes enters epithelial and other non-phagocytic cells including macrophages by inducing their own endocytosis in a phenomenon called parasite-directed endocytosis (Racz, Tenner & Mero, 1972). The signalling events which lead to bacterial engulfment through parasite-directed endocytosis remain unclear. In the case of *L. monocytogenes* it has been suggested that the signalling events are probably triggered by internalin binding to E-cadherin (Mengaud *et al.*, 1996).

After entering non-microbicidal cells *L. monocytogenes* is internalized into phagocytic vacuoles (Gaillard *et al.*, 1987). It appears that following phagocytosis the

release of haemolysin and phospholipase C breaks down the phagosome vacuole membrane which subsequently allows the organism to enter the cell cytoplasm, where multiplication is improved by more-favourable growth conditions (Portnoy, Jacks & Hinrichs, 1988). The bacterium then becomes coated with host actin, which polarizes (F-actin) and through interaction with components of the host cell cytoskeleton, the bacterium is propelled from the first infected cell into an adjacent cell and into the blood and lymph systems (Mounier *et al.*, 1990). *L. monocytogenes* avoids detection by circulating antibodies by entering phagocytic cells such as macrophages, fibroblasts and hepatocytes where the bacterium multiplies and again moves to adjacent cells. The bacterium can, therefore, be effectively disseminated within infected enterocytes to all body areas and avoid natural host defence systems. A review of the intracellular multiplication and movement of *L. monocytogenes* in host macrophages is given by Tilney & Tilney (1993).

1.4.3 Groups at increased risk from *Listeria* infection.

Healthy individuals appear to be resistant to *L. monocytogenes* infection as indicated by the relative infrequency of clinical disease in such individuals (McLauchlin, 1987). Certain groups of people within the population seem to be at increased risk from *L. monocytogenes* and acquired susceptibility to infection in humans does exist. There are several host factors which predispose humans to listeriosis. These are:-

1.4.3.i Immunosuppression.

Both healthy animals and humans overcome listeric infection by virtue of cell-mediated immunity (El-Kest & Marth, 1988a). T-lymphocytes in healthy individuals activate macrophages to kill *L. monocytogenes*. The majority of human cases of listeriosis occur in individuals who have an underlying condition which leads to a depressed level of T-lymphocytes (El-Kest & Marth, 1988a). Such predisposing conditions often associated with listeriosis include pregnancy, extremes of age, drug induced immunosuppression, cancer, AIDS, congenital immune deficiencies and diabetes mellitus (Nieman & Lorber, 1980).

In a recent summary of listeriosis cases in 1989 from 16 countries, it was shown that 31 and 22% of total cases occurred in patients older than 60 years and younger than 1 month respectively (Rocourt, 1991).

1.4.3.ii Achlorhydria.

Gastrointestinal immunity to invasive infection has been shown to play a non-specific protective role in killing ingested organisms by the action of gastric acid (Ho *et al.*, 1986). In patients with achlorhydria, hydrochloric acid production is reduced and thus individuals are pre-disposed to infection.

1.4.3.iii Hyperferraemia (excess of iron in the blood).

The lack of free iron in the host environment provides a non-specific method to prevent microbial growth in the host. In humans with clinical conditions which result in hyperferraemia (excess iron), susceptibility to *L.monocytogenes* infection is, therefore, increased.

1.4.4 Clinical syndromes of listeriosis.

L. monocytogenes is responsible for three major clinical syndromes in humans, namely, meningoencephalitis, septicemia, and intrauterine infection of the foetus (Schaffner, 1986). Rocourt (1991) reported that from a total of 782 cases of listeriosis from 16 countries in 1989, 43% were maternal and neonatal infections, 29% were septicemic infections, 24% were central nervous system infections and 4% were atypical forms.

1.4.4.i Meningoencephalitis.

Meningoencephalitis is an inflammation of the brain and meninges and is found mainly in the elderly and immunocompromised patients (Farber & Peterkin, 1991). Mortality rates among untreated patients may be as high as 70% (Seeliger & Finger, 1976), and about 30% for all cases (Poyart-Salmeron *et al.*, 1990). *L. monocytogenes* accounts for 1-2% of cases of adult meningitis and is the third most common cause of

meningitis in neonates after *Escherichia coli* and Group B streptococci (Lorber, 1990).

1.4.4.ii Septicemia.

Septicemia is a blood infection and is the most common listeric manifestation in adults and immunocompromised hosts where mortality rates of 30% have been reported (Fleming *et al.*, 1985). In this stage of infection the organism has access to all body areas and may involve the central nervous system, heart, eyes or the foetus of pregnant women.

1.4.4.iii Intrauterine infection of the foetus.

Maternal listeriosis does not inevitably lead to infection of the foetus, as has been noticed in several instances where only one case of infant listeriosis has been occurred in a twin delivery (Farber & Peterkin, 1991). Healthy pregnant women may be carriers of *L. monocytogenes* and still give birth to healthy infants. However, the stage of pregnancy when exposed to the organism determines the outcome of the foetus (abortion, stillbirth or neonatal sepsis).

Two clinical forms of neonatal listeriosis, early- and late-onset forms, are known. The mean incubation time for the onset of symptoms of the former disease is 1.5 days and results from intrauterine infection. The clinical presentation of early-onset disease is most often sepsis although widespread disseminated granulomas are sometimes found in the liver and placenta (Schuchat *et al.*, 1991). Late-onset listeriosis occurs several days or weeks after birth, with meningitis as the predominant form of the disease. The source of the organism in these late-onset cases is usually unclear. However, the infection may be acquired either from the mothers genital tract during birth or from environmental sources after birth (Vandepitte & Ruelens, 1988). Case fatality rates appear to be lower in late-onset disease than in early-onset infection. In a retrospect series reported from Britain between 1967 and 1985, late-onset disease had a mortality rate of 26% while early-onset disease had a case fatality rate of 51% (McLauchlin, 1990).

The early diagnosis and provision of effective antibiotic treatment are crucial for

the survival of neonates with listeriosis (Farber & Peterkin, 1991). The treatment of choice is benzylpenicillin or ampicillin combined with an aminoglycoside antibiotic (Chen *et al.*, 1996).

1.4.4.iv Atypical forms of listeriosis.

Additional forms of listeriosis are rare but include pneumonia, endocarditis of native or prosthetic valves, endophtalmitis and osteomyelitis, with the involvement of sites such as the skin, spleen, gall bladder and lymph nodes (Schlech, 1991).

McLauchlin & Low (1994) reviewed seventeen cases of cutaneous listeriosis in humans. This condition presented itself as vesicular or pustular lesions usually on the arms or hands. Sixteen of the 17 reported cases involved farmers or veterinary surgeons and occurred most often after the manual delivery of aborting bovine fetuses or still births. These case studies provide evidence of zoonosis, i.e. a human infection acquired directly from animals.

1.5 Epidemiology of listeriosis.

The epidemiology of *L. monocytogenes* is still poorly understood. This is particularly due to the sporadic nature of most of the outbreaks (Gellin & Broome, 1989) but also because serological typing methods are not sufficiently discriminating and many strains are not phage typable (McLauchlin *et al.*, 1986; Bibb *et al.*,1990). As previously described in Section 1.2.2, methods for typing *Listeria* species have improved recently with the development of systems such as multi-locus enzyme electrophoresis. Consequently, future epidemiological studies will be more detailed than those that currently exist and may provide better information linking sporadic cases to common-source outbreaks.

1.5.1 Sporadic incidence of listeriosis in the United Kingdom.

During the last decade several articles reporting case series and prospective and retrospective surveillance of listeriosis showed significant upward trends in the incidence of this disease (Schlech, 1991). Fewer than 100 cases of listeriosis were reported

annually to the Public Health Laboratory Service (PHLS) in England, Wales and Northern Ireland between 1967 and 1982. Numbers of cases then increased, slowly at first, from 115 in both 1983 and 1984, 149 in 1985, and 137 in 1986. A near doubling of cases then occurred to 259 in 1987, 291 in 1988, and 250 in 1989. Subsequently, only 90 cases were reported in the first nine months of 1990 (McLauchlin *et al.*, 1991). In Scotland, Campbell (1990) recorded cases of listeriosis from 1967 to 1988 noting an increase in the incidence from 0.5 per million to 7 per million between 1987-1988. Mother-infant case pairs were the most common (64%) source of infection.

The upsurge in cases of listeriosis in the UK between 1985 and mid 1989 was caused largely by two strains of *L. monocytogenes* (serotype 4b phage type 6,7 and serotype 4bX) which accounted for 30-54% of the annual totals (McLauchlin *et al.*, 1991). These strains were less common before 1987 and after July 1990. A survey of patés in England and Wales in July 1989 showed that they frequently contained *L. monocytogenes*. A similar survey in July 1990 showed a reduction in the proportions of contaminated paté samples (McLauchlin *et al.*, 1991). In 1989 patés from a single plant (manufacturer Z) were found to be more likely to be contaminated with *L. monocytogenes* and at higher levels than those from other producers. Most strains of *L. monocytogenes* recovered from manufacturer Z paté in 1989 were indistinguishable from those responsible for the 1987-1989 upsurge in human listeriosis. Additionally, these isolates were uncommon among isolates in patés from other manufacturers and from a wide range of other foodstuffs (McLauchlin *et al.*, 1991). Furthermore, patients infected with the types of *L. monocytogenes* found in paté were significantly more likely to have recently eaten paté than those affected by other strains. The start of the decline in the number of cases of listeriosis coincided with a government health warning on paté consumption (ANON, 1989) and the suspension of supplies from manufacturer Z (McLauchlin *et al.*, 1991). From the above facts it was concluded that contamination of paté was a likely contributory cause of the increase in the incidence of listeriosis in the UK between 1987 and 1989 (McLauchlin *et al.*, 1991).

It should be noted that the above evidence was circumstantial; there was no direct microbiological evidence linking cases with the consumption of paté, as no

samples of paté consumed by any of the patients were available for examination (McLauchlin *et al.*, 1991).

Analysis of the seasonal pattern of listeriosis shows a consistent increase in cases of listeriosis during the late summer to early autumn period (McLauchlin, 1990). Such seasonal peaks occurred in 1986 and 1987, in addition to winter peaks in 1985 and 1987. In the first nine months of 1990 a seasonal pattern was absent. Cox (1989) suggested this seasonality could be due to the influence of climatic factors on the general susceptibility to disease. Alternatively, this seasonality could be due to the presence of other seasonal infections such as *Salmonella enteritidis* Pt4 which also increased by over 100% from 1985 to 1989 and possibly predisposed people to infection with *L. monocytogenes* (Cox, 1989).

1.5.2 Sporadic incidence of listeriosis in Europe and the United States.

The incidence of listeriosis on the European continent also appears to have increased in recent years. Rates of listeriosis in Europe vary tenfold with highest reported frequencies in Belgium, East Germany and France (Schlech, 1991). A study from Denmark by Samuelsson *et al.* (1990) recorded an incidence between 1958-1984 of 2.3 per million per year. During the course of an 'outbreak' in 1985-1987 the incidence peaked at 8.4 per million. Since this outbreak, *Listeria* infections in Denmark have continued to occur in higher numbers (around 6 to 7 cases per million per year) than at the beginning of the 1980s (Nørrung & Skovgaard, 1993).

In an intensive surveillance of cases in six scattered geographical areas of the US, an incidence of 7.1 per million was calculated for 1986 (Gellin *et al.*, 1991). Highest rates were recorded in the perinatal period (124 cases per million) rather than in adult cases (5.4 cases per million).

Increased awareness of listeriosis and more effective reporting of its incidence may have contributed to the increase in numbers of cases reported over recent years. However, Schlech (1991) commented that the overall impression developed from reviewing surveillance data is that the incidence of sporadic listeriosis has increased significantly over the last decade.

Although the source of sporadic listeria infection is rarely determined (Schuchat *et al.*, 1991) a number of large common-source outbreaks have been directly linked to the consumption of contaminated food.

1.6 Evidence for food-borne transmission of *Listeria* to animals and man.

It is 60 years since it was first suggested that *L. monocytogenes* may be transmitted to humans through the consumption of contaminated food (Burn, 1936) and over 30 years since a relationship between the feeding of silage and listeriosis was demonstrated in animals (Gray, 1963) (Section 1.6.1). It was not, however, until several reports of food-borne outbreaks due to *L. monocytogenes* in the 1980s (Section 1.6.2) that interest in this mode of transmission to humans increased dramatically.

1.6.1 Transmission to animals.

L. monocytogenes infection in ruminants has frequently been reported to occur concurrent with silage feeding (Gray, 1960, 1963; Grønstøl, 1979; Fenlon, 1986ab).

Grass silage is an important winter feed for ruminants and is the product of a natural lactic acid bacterial fermentation under anaerobic conditions. *L. monocytogenes* is often present in very low numbers in grass used for ensiling and these bacteria have been shown to persist in silage for 10-12 years (Dijkastra, 1975). Poor-quality silage, particularly that having a high pH, is more likely to yield *L. monocytogenes* (Grønstøl, 1979). *L. monocytogenes* can proliferate in poor quality silage reaching levels $> 10^6$ g^{-1} (Fenlon, 1986b). The presence of significant numbers of *L. monocytogenes* in silage occurs when anaerobic conditions are not strictly maintained throughout the ensiling period (Woolford, 1990).

Recent increases in listeriosis observed in sheep and cattle have been related to the increased use of big bale silage in preference to conventional silage (Fenlon, Wilson & Weddell, 1989). Big bale silage is more susceptible to aerobic spoilage and consequently to *L. monocytogenes* growth than conventional clamp silage (Fenlon *et al.*, 1989; Donald, Fenlon & Seddon, 1995). In big bales both the wrapping process and the high surface area to volume ratio increases the potential for air infiltration and the

creation of suitable growth conditions for *L. monocytogenes* (Muck, 1993). Donald *et al.* (1995) demonstrated that *L. monocytogenes* survival depends on establishing a fine balance between the physico-chemical and microbial characteristics of the silage, i.e, oxygen tension, dry matter, pH, grass type and chemical and microbiological quality.

1.6.2 Transmission to humans.

Since 1979, several outbreaks of listeriosis have occurred in humans where food was clearly implicated as the means of transmission of the bacterium. Two of these outbreaks incriminated salad vegetables as a common-source of infection.

The first reported case study where a salad vegetable was implicated as a vector was reported by Schlech *et al.* (1983). This case occurred in a maternity hospital in Nova Scotia, Canada and provided the first evidence for transmission of listeriosis by the food-borne route. The outbreak was large, with 7 adult and 34 perinatal cases of listeriosis reported between March and September 1981. These included 5 spontaneous abortions and 4 still-births. A case-control study conducted during the investigation suggested that patients were more likely than controls to have eaten a raw vegetable dish (coleslaw) prior to their infection. Coleslaw from one patient refrigerator was contaminated with *L. monocytogenes* 4b, the same serotype as the epidemic strain. The coleslaw had been prepared locally and two unopened packages of coleslaw from the manufacturer also contained the organism. Further investigations showed that cabbage included in the coleslaw was grown in fields fertilised with both raw and composted manure from a flock of sheep in which there had been three recent deaths from listeriosis. It was subsequently implied that the pH of the coleslaw was higher than 5.0, Today coleslaw prepared commercially in the U.K. contains sufficient acetic acid to reduce the pH to 4.5 (Lund & Lyon, 1990).

The second reported case in which raw salad vegetables were implicated as the source of infection occurred in eight Boston hospitals in 1979 (Ho *et al.*, 1986). In the outbreak, 20 patients admitted to hospitals in the Boston area had systemic *L. monocytogenes* infection. All isolates were *L. monocytogenes* type 4b, only nine of the isolates serotyped during the preceding 26 months had been 4b, indicating an epidemic.

Of these twenty case patients, 12 had received antacids or cimetidine (a drug used to reduce gastric acid production) before the onset of listeriosis. A food-preference study indicated that three foods were preferred by case patients more frequently than by control patients: tuna fish, chicken salad and cheese. However, the only commonality appeared to be the serving of these foods with raw celery, tomatoes and lettuce. On this evidence it was suggested that these raw vegetables were contaminated with *Listeria*, which was able to survive ingestion because of gastric acid neutralisation.

Following these two outbreaks, Bendig & Strangeways (1989) documented a further incidence of human listeriosis where contaminated lettuce was implicated as the source of hospital-acquired listeriosis. A 74-year old man at St James Hospital, London who had received cimetidine for the treatment of stomach ulcers developed the disease. In an examination of ready-to-eat foods prepared in their hospital kitchen including dairy products, *L. monocytogenes* was only isolated from washed English round lettuce. Although there was no direct proof that the patient had acquired *L. monocytogenes* from the hospital lettuce, it was emphasised that merely washing the lettuce did not make it safe for consumption by immunocompromised patients.

Three cases of listeriosis have implicated dairy products as a means for *L. monocytogenes* transmission to humans. The first of these cases occurred in Massachusetts, USA, where 49 people were affected by listeriosis between June and August 1983 (Fleming *et al.*, 1985). Seven cases occurred in foetuses or infants and 42 in immunosuppressed adults. A link was demonstrated between many of the cases and the consumption of pasteurised milk produced in a single factory. *L. monocytogenes* was isolated from raw milk entering the plant, and because the pasteurisation process was believed to be satisfactory, it was concluded that the outbreak probably resulted from raw milk contaminating pasteurised milk.

A similar outbreak of listeriosis occurred in Los Angeles county, California between January and August 1985. One hundred and forty two cases of human listeriosis were reported (Linnan *et al.*, 1988). Epidemiological studies linked the outbreak with a brand of Mexican style soft cheese. It was concluded that some of the milk used in production of the cheese had probably not been pasteurised. This outbreak

was interesting because 87% of the pregnancy-associated cases occurred in a single ethnic group (Hispanic). The patients were principally cared for at a single medical facility. Consequently the outbreak was quickly recognised and the implicated food recalled. This outbreak highlights that many apparently sporadic cases of listeriosis may be the result of a single contaminated food source. However, temporal clustering may not be recognised if the vehicle is widely distributed or if patients present to different medical facilities (Schuchat *et al.*, 1991). In addition, this study also reported that the interval between eating contaminated food and the onset of symptoms was up to 70 days. This is markedly longer than that seen in other food-borne diseases which may cause symptoms after only a few hours. This obviously complicates epidemiological investigations since relevant food exposures may be those which occurred several weeks prior to the onset of disease (Schuchat *et al.*, 1991).

Between 1983 and 1987 an epidemic of 122 cases of listeriosis occurred in the Canton of Vand in Switzerland. The outbreak was attributed to infected Vecherin Mont d'Or cheese. The environment in the ripening cellars was found to be heavily contaminated with *L. monocytogenes* and led to a worldwide recall of the cheese (Bille & Glanser, 1988).

Other common-source outbreaks of listeriosis have implicated cook-chilled chicken (Kerr, Dealler & Lacey, 1988), jellied pork tongue and pork paté (Goulet, 1995). The latter two cases occurred in France in 1992 (279 cases) and 1993 (39 cases) respectively. The 1992 outbreak resulting in 63 deaths and 22 miscarriages. The rapid identification of the vehicle in the 1993 outbreak with its immediate recall and warnings to pregnant women by the public health authority reduced the number of cases below the expected level.

In February 1988 a World Health Organisation (WHO) working group concluded that '*L. monocytogenes* is an environmental micro-organism whose primary means of transmission to humans is through foodstuffs contaminated during production and processing' (ANON, 1988a). The group suggested that research should be directed to determine ways in which *L. monocytogenes* can be eliminated from the raw food supply.

1.7 Incidence of *Listeria* in food.

Since the 1980s outbreaks of food-borne listeriosis, many studies have examined the incidence and growth of *L. monocytogenes* in a wide variety of food samples.

1.7.1 Incidence of *Listeria* in milk, dairy products, pate and meat samples.

Farber, Sanders & Johnston (1989b) and Lund & Lyon (1990) summarised the results of several surveys in which *Listeria* had been isolated from food samples. These reports indicated that the organism could not be isolated from pasteurised milk, brie cheese from pasteurised milk, cooked prawns, shrimps, cockles and deserts, but was isolated from up to 5% of raw milk, up to 5.5% of ice cream, 1 to 10% of sort-ripened cheese, 10% of brie cheese from raw milk, up to 20% of fermented sausage, 16% of salami, 10% of chilled paté, 51% of paté, 30 to 50% of raw meats, 30 to 100% of ground meats, and 14 to 80% raw poultry and chicken.

The relatively high rates of *Listeria* in unpasteurised milk, coupled with recent milk related outbreaks of listeriosis, demonstrates that enforcement of regulatory guidelines for pasteurisation procedures and the elimination of sources of post-pasteurisation contamination is of vital importance. High frequency of *Listeria* in meat and poultry samples has been attributed to faecal contamination in the rearing environment, in addition to cross contamination from abattoirs or poultry-packing plant effluent (Farber *et al.*, 1989b; Watkins & Sleath, 1981). The increased incidence of *Listeria* in ground meat samples compared to raw meat samples may be attributed to the additional handling and processing of these food stuffs.

1.7.2 Incidence of *Listeria* in individual and mixed salad vegetables.

L. monocytogenes has been isolated from minimally processed fresh vegetables at frequencies which vary from 0 to 30% of samples (Lainé & Michard, 1988; Sizmur & Walker, 1988; Heisick *et al.*, 1989; Farber *et al.*, 1989b; Velani & Roberts, 1991; Beufort, Poumeyrol & Rudelle, 1992; Breer & Baumgartner, 1992).

Between October 1987 and August 1988, Heisick *et al.* (1989) tested approximately 100 samples of each of 10 types of fresh fruit and vegetables. *Listeria*

spp. were isolated from 0% of broccoli, carrots, cauliflower, tomatoes, 1.1% of lettuce, 2.2% of cabbage, 10.9% of cucumbers, 12% of mushrooms, 25.8% of potatoes and 30.3% of radishes. It was speculated that broccoli, cauliflower and tomatoes were less contaminated because they have less contact with the soil than the root crops. Overall, 48% of isolates were *L. monocytogenes*.

Farber *et al.* (1989b) sampled 50 lettuce, 30 celery, 10 radish and 20 tomato samples for *Listeria* spp. None of the vegetables analyzed were found to contain *L. monocytogenes* and in only 1 (radish) of the 110 samples were any *Listeria* spp. recovered. The authors concluded that *Listeria* spp. may have been present on their vegetable samples, but at levels too low to detect. The minimum detection level was not, however, stated.

Sizmur & Walker (1988) examined 60 mixed salads obtained from supermarkets and isolated *L. monocytogenes* from 4 samples representing two salad types. These salad types were unrelated in contents. The first consisted of cabbage, celery, sultanas, onion and carrot whilst the second consisted of lettuce, cucumber, radish, fennel, watercress, and leeks. *Listeria* spp. were not isolated from the plain beansprout salads or from those which contained nuts. It was suggested that this may have been due to the acidic pH of these salads.

A more detailed study of 42 prepacked mixed salads and 108 individual salad ingredients was carried out by Velani & Roberts (1991). *L. monocytogenes* was isolated from 10 of the 150 (6.7%) salad samples. Of the 42 prepacked mixed salads, eight (19%) contained *L. monocytogenes* while only two of the 108 (1.8%) individual salad ingredients contained the organism. It was suggested that the high level of *Listeria* in mixed salads was probably due to the introduction and further spread of contamination during the processes of chopping, mixing and packaging during preparation. Levels of *L. monocytogenes* in all samples tested were <200 per gram.

1.8 Growth of *L. monocytogenes* on salad vegetables.

In recent years the demand for convenience food has dramatically increased the consumption of commercially prepared, ready-to-eat fresh salad vegetables. These

vegetables, whether offered for sale in retail groceries, restaurant salad bars or in fast food operations, are kept under refrigeration to maintain sensory quality. Although levels of *L. monocytogenes* contamination of individual salad ingredients are usually low (< 200 cells per gram) (Section 1.7.2), the ability of *L. monocytogenes* to survive and grow at refrigerated temperatures increases the potential for these organisms to establish at potentially lethal concentrations in these products. These concerns are increased when whole or ready-to-serve salad vegetables are held at salad bar ambient temperatures for extended periods prior to consumption (Ryser & Marth, 1991). Several workers have therefore, investigated the survival and/or growth of *L. monocytogenes* in vegetable juices and on a range of individual salad ingredients (Sections 1.8.1 to 1.8.5).

Most studies which have examined the growth/survival of *L. monocytogenes* on whole leaf vegetables have used a dipping procedure to artificially inoculate individual leaf pieces prior to incubation. Typically, bacterial cells are grown in a broth culture such as Tryptone Soy broth and then resuspended in distilled water to concentrations which range from 10^3 to 10^5 cfu ml^{-1}. Following dipping, leaf pieces are drained and incubated within sealed plastic bags and viable cell counts determined from leaf macerates over time. Although this system has the benefits of allowing easy inoculation and storage of samples, it should be noted that both the cut edges of leaf samples and the leaf surface itself is always inoculated. Thus, depending on the size, thickness, area of cut tissue exposed and indeed the type of leaf tissue under study, large variability (even within one experiment) has often been reported in these investigations making comparison between such studies difficult. This was illustrated to some extent by Beuchat & Brackett (1990a) who showed that shredded lettuce when dipped in *L. monocytogenes* retained more cells than whole lettuce which had a smaller surface to weight ratio.

1.8.1 Growth on lettuce.

Steinbreugge, Maxcy & Liewen (1988) illustrated that *L. monocytogenes* grew in Iceberg lettuce juice at 5°C, multiplying from 7.2 x 10^3 to 9.9 x 10^7 cfu ml^{-1} between 0 and 14 days after inoculation. Additionally, *L. monocytogenes* grew on pieces of

shredded Iceberg lettuce tissue stored at both 5 and 12°C (Steinbreugge *et al.*, 1988). Cell concentrations reached 10^5 cfu g^{-1} after 7 d incubation and 10^6 cfu g^{-1} after 14 d at both temperatures. However, this represented slightly over one $\log_{10}$ growth in 14 d at 5°C and slightly over three logs growth at 12°C in 14 d indicating that the rate of multiplication was larger at the higher storage temperature. It should be noted that in additional trials the organism did not grow on lettuce tissue or had died after 14 days. The variation in results between replicate experiments could not be fully explained, but was partially attributed to differences in initial inoculum concentration, pH, and competition from the natural bacterial flora. Beuchat & Brackett (1990a) also examined the growth of *L. monocytogenes* on Iceberg lettuce. They also found that growth kinetics were largely dependent on storage temperature. At 10°C significant increases occurred within three days and reached 10^8-10^9 cfu g^{-1} by 10 d, whereas at 5°C significant increases did not occur until 8 d and continued to increase to 15 d when the trial was terminated. Populations at 15 d for the 5°C incubation temperature were, however, lower than those at 10 d for the higher incubation temperature.

Lettuce type has been shown to influence growth characteristics of *L. monocytogenes*. Carlin & Nguyen-the (1994) observed that growth patterns of *L. monocytogenes* on isolated leaves of Broad-leaved endive, Curly-leaved endive, Lamb's lettuce and Butterhead lettuce over a 7 d incubation period at 10°C were markedly different. During the 4 d storage period, cell populations increased by 1.5 $\log_{10}$ on butterhead lettuce, by 0.5 $\log_{10}$ on curly-leaved and broad-leaved endives and decreased by about 1 $\log_{10}$ on Lamb's lettuce. Hence, the risk of intoxication by consuming contaminated salads increases with storage time for Broad-leaved endive, Butterhead lettuce and to a lesser extent, Curly-leaved endive, but not for Lambs lettuce. It was noted that these differences on the four salads were not related to the population of epiphytic bacteria present throughout the incubation period. Growth patterns of epiphytic microflora were more comparable among the four salad types than *L. monocytogenes*, and mean growth of epiphytic microflora was higher than that of *L. monocytogenes*.

In an extended study using Broad-leaved endive, Carlin, Nguyen-the & Abreau da Silva (1995) illustrated that growth of *L. monocytogenes* was dependent on the

incubation temperature, inoculum concentration, leaf age and extent of leaf spoilage. As observed with Iceberg lettuce (Steinbreugge *et al.*, 1988; Beuchat & Brackett, 1990a), growth rates of *L. monocytogenes* and resident aerobic bacteria on Broad-leaved endive were significantly reduced when storage temperature was decreased from 20°C to 3°C (Carlin *et al.*, 1995). A low inoculum of *L. monocytogenes* showed a faster increase in populations than a high inoculum, but despite this, the plateau reached at between 4 and 7 d of storage at 10°C by the populations of *L. monocytogenes* was lower than that for high inoculum. This result contrasts with other work done in broth (Buchanan & Phillips, 1990) or on poultry product (Wimpfheimer, Altman & Hotchkiss, 1990), where it was concluded that growth kinetics of *L. monocytogenes* were not affected by the size of the inoculum.

On Broad-leaved endive, the highest increases in *L. monocytogenes* populations were recorded on leaves exhibiting extensive spoilage (Carlin *et al.*, 1995). This was possibly due to increased nutrient availability on such samples compared to healthy tissue. Additionally, populations of *L. monocytogenes* increased more rapidly on pieces of young yellow leaves than on pieces of older green leaves. This difference was not, however, related to the number of aerobic mesophyllic bacteria or the extent of spoilage of the leaves and no clear conclusions were drawn to explain this observation (Carlin *et al.*, 1995). Interestingly, the authors calculated that an initial population of 10 cfu *L. monocytogenes* g^{-1} on leaves of endive could increase to 5 x 10^4 cfu g^{-1} after storage of leaves at 10°C for 4 d, without extensive spoilage. They commented that the limit of 100 *L. monocytogenes* g^{-1} in minimally processed vegetables at the processing level suggested by Farber (1993) and recommended in France (ANON, 1993a), can only be achieved by using a strictly controlled chain of refrigeration at processing, storage and retail with temperatures never exceeding 3-4°C.

1.8.2 Growth on cabbage.

L. monocytogenes was shown to grow on raw shredded cabbage during the first 25 days of a 64 d incubation period at 5°C (Beuchat *et al.*, 1986). Populations increased from 1.6 x 10^4 to 2.6 x 10^8 cfu g^{-1} from 0 to 25 d. Extended storage from 25 to 64 days

resulted in only a slight decrease in the viable population. In contrast *L. monocytogenes* cell populations declined in heat-sterilised cabbage stored at 5°C for 42 d. This suggests that heat treatment either decreased the availability of certain nutrients or increased constituents which inhibited growth or were toxic (Beuchat *et al.*, 1986).

The survival of *L. monocytogenes* on discs of cabbage leaf tissue was examined over a 7 d incubation period at 10°C by Wilson (1994). Wilson observed that cell populations remained relatively stable throughout the incubation period. It should be noted that in this study leaf tissue was inoculated by placing a drop of inoculum onto the epidermal leaf surface and therefore cut leaf surfaces were not inoculated. Additionally, a very high inoculum concentration of 1 x 10^6 cfu per disc was used to inoculate cabbage tissue. In light of these observations, it is not surprising that *L. monocytogenes* populations did not increase as dramatically as those observed by Beuchat *et al.* (1986).

1.8.3 Growth on asparagus, cauliflower and broccoli.

Berrang, Brackett & Beuchat (1989) showed that *L. monocytogenes* could grow on asparagus, cauliflower and broccoli samples. At storage temperature of 15°C, viable cells increased rapidly on all vegetable types. Increases from 10^4-10^5 to 10^7-10^8 cfu g^{-1} on asparagus within 6 d, from 10^5-10^6 to 10^8-10^9 cfu g^{-1} within 10 d on broccoli, and from 10^3-10^4 to 10^6-10^7 cfu g^{-1} within 8 d on cauliflower were recorded. At 4°C the increases were less marked; on broccoli and cauliflower viable cells remained relatively stable over 21 d of storage but increased by 2 $\log_{10}$ on asparagus over 21 d.

1.8.4 Growth on tomatoes and in tomato products.

Beuchat & Brackett (1991) demonstrated that growth of *L. monocytogenes* on whole tomatoes occurred when they were incubated at 21°C (increasing from approximately 10^3 to 10^5 cfu g^{-1} within 2 d) but not at 10°C. In contrast, death occurred in chopped tomatoes held at these temperatures (the rate of death at 10°C was slower than at 21°C). Death was attributed to the presence of acetic acid in the tomatoes. It was concluded that unlike low-acid raw salad vegetables such as lettuce on which *L.*

monocytogenes has been shown to grow at refrigerated temperatures, tomatoes are not a good growth substrate for the organism. Nevertheless, *L. monocytogenes* can remain viable on raw whole tomatoes and chopped tomatoes for periods extending beyond their normal shelf life expectancy. Beuchat & Brackett (1991) also showed that the survival of *L. monocytogenes* inoculated into commercially processed tomato juice and sauce was dependent on storage temperature. Surprisingly, when held at 5°C, populations remain constant for 14 days whereas a gradual decrease in the number of viable cells was observed in juice and sauce held at 21°C. Additionally, the organism died rapidly in tomato ketchup at both 5°C and 21°C. This was probably due to the higher acetic acid content in ketchup compared to juice and sauce which results from dehydrating this product to form a concentrate.

1.8.5 Growth on carrots.

As detailed in section 1.7.2, Heisick *et al.* (1989) failed to detect *L. monocytogenes* on any carrot samples examined. Interestingly, Beuchat & Brackett (1990b) demonstrated that whole carrots dipped in *L. monocytogenes* cells became contaminated with higher populations of viable cells than shredded carrots treated in the same way. This was surprising as one might predict the opposite; i.e. large populations would be detected on shredded carrots, since the surface area/weight ratio was higher than for whole carrots. It was suggested that naturally occurring constituents or phytoalexins in cellular and vascular fluids, which may be released as a result of rupturing carrot cells, had a toxic effect on *L. monocytogenes*. The toxic component in carrots is reduced or rendered insoluble or inactive upon exposure to heat. This was concluded from the observation that cooked carrots dipped in cell suspensions did not have any effect on cell viability. Beuchat & Brackett (1990b) also demonstrated that small populations detected on whole carrots immediately after dipping were non-detectable after 7 d storage at 5°C or 15°C.

Evidence of the lethal effect of carrot on nine strains of *L. monocytogenes* and single strains of *L. innocua*, *L. ivanovii*, *L. seeligeri* and *L. welshimeri* was presented by Nguyen-the & Lund (1991). Viable cell populations on commercially prepared

shredded carrots fell by up to 3 $\log_{10}$ in the first 3 d of storage at 8°C.

Recently, Babic *et al.* (1994) investigated the active antimicrobial constituent of carrot juice. They illustrated that the phenolic phytoalexin of carrots, 6-methoxymellein, although having antimicrobial effects against a range of food-borne microorganisms when present in high concentrations, did not accumulate to sufficiently high concentrations in shredded carrot to have antimicrobial activity. Filtration of carrot extracts through a C_{18} Sep Pack column removed their antimicrobial activity, suggesting an apolar nature of the antimicrobial components. Free saturated fatty acids (dodecanoic acid) and methyl esters of saturated fatty acids (of dodecanoic and pentadecanoic acids) were identified in purified active extracts by gas chromatography coupled to mass spectrometry and could be responsible for the antimicrobial activity (Babic *et al.*, 1994).

1.9 Concepts for the control of *Listeria* in food processing environments.

The contamination of food products may lead to illness or death of a consumer, product recalls with their associated adverse publicity resulting in loss of sales and profits. If regulatory requirements have been infringed, fines, sanctions, loss of export licence or ultimately site closure may ensue (Holah *et al.*, 1994). To avoid such events and to fulfil their commitment to public welfare, food manufacturers devote significant resources to ensure the production of safe food products (ANON, 1993b). For many years emphasis on end-product testing of food samples was used predominantly to control the safety of the products on the market (van Schothorst & Jongeneel, 1994). However, four main problems of end-product testing exist. Firstly, contaminants are frequently not distributed homogenously throughout a product and consequently may not be detected when the sample size is small. Secondly, even if the distribution is homogenous, it is difficult to test a sufficient number of sample units to obtain meaningful information. Thirdly, many tests are time consuming, expensive and hamper the quick release of products. Finally, end product testing detects only effects; it neither identifies nor controls the cause of contamination (van Schothorst & Jongeneel, 1994).

Regulators and food business operators have recently placed increasing emphasis on the 'Hazards Analysis of Critical Control Points' (HACCP) concept to assure that

food products are safe when eaten (van Schothorst & Jongeneel, 1994). Rather than relying on end-product testing, HACCP looks at the whole food manufacturing line to identify the points where problems may occur and be prevented or minimized, and 'monitors' the control measures. The accepted definition of the HACCP concept is 'a systematic approach to the identification and assessment of the microbiological hazards and risks associated with the manufacture, distribution and use of a particular foodstuff, and the definition of means for their control' (ANON, 1988b). ANON (1988b) include in their definition of a hazard: 'the unacceptable contamination, unacceptable growth, and/or unacceptable survival by microorganisms of concern to safety'. Microbial control is thus the prevention of (re)contamination, growth, and survival to the extent deemed necessary. The substrate for growth etc. is primarily the product at any stage of production, but growth and survival on the production line (in food residues or biofilms [see Sections 1.10.2 & 1.12 for detailed description of biofilms]) must also be controlled, because product and line cannot be separated in many cases. Additionally, growth or survival in the direct environment of an open line should also be considered. Unacceptable growth in such an environment can be regarded as a source of hazard (ANON, 1992).

The initial development and application of the HACCP technique was first presented in by the American Public Health Association in 1972 (ANON, 1972). Since then HACCP has been applied internationally in food manufacturing sectors (Bauman, 1974; Munce, 1984; Bryan, 1990; ANON, 1993bc; Kirby, 1994; Ali & Spencer, 1996; Todd & Harwig, 1996).

The HACCP analysis system consists of six steps (ANON, 1988b): 1) The identification of hazards and assessment of the severity of those hazards and their risks associated with the growing, harvesting, processing or manufacture, distribution, preparation and/or use of a raw material or final product. 2) Determination of critical control points (CCPs) at which hazards can be controlled. A CCP may be a raw material, or a location, practice, procedure or process at which control can be exercised over one or more factors, thus preventing a hazard. 3) Specification of criteria (i.e. limits and tolerances) that indicate whether an operation is under control at a CCP. 4)

Establishment and implementation of procedures to monitor that each CCP operates under control. 5) Identification of any corrective action required when a CCP operates 'out of control'. 6) Verification. i.e. the use of additional information to ensure that the HACCP system is working.

Many commercial food processes involve over 50 different stages from raw material production or acquisition to final product use. HACCP analysis allows management to identify the stages (typically between five and ten CCPs) that are critical to the safety of the product and, therefore, allows managers to concentrate technical resources to ensure that the critical operations are adequately controlled (Kirby, 1994). Criteria for selection of CCPs may differ considerably depending on the product under manufacture. For example, in a cooked meat product it is possible to eliminate a pathogen during the cooking process. Thus, the goal in establishing CCPs for biological hazards for cooked products would be to eliminate contaminants and to prevent their reintroduction following cooking. For a product such as ground raw beef, pathogens can not be eliminated if present and, therefore, the goal of HACCP is to minimise the possibility of contamination with pathogens and minimise their potential for growth (ANON, 1993c).

The control of microbial hazards thus deals with the prevention or limitation of (re)contamination, growth and survival. Possibilities for (re)contamination (Section 1.10) depend to a large extent on the amount of growth in residues and on food contact surfaces (Section 1.10.2) and the efficiency of cleaning procedure used (Section 1.11). Growth and survival depend on parameters such as: temperature, time, pH, a_w and preservative (Section 1.11). Finally, HACCP only becomes fully effective when the consumer accepts that they also have a role to play in the prevention of food-borne disease (van Schothorst & Jongeneel, 1994).

1.10 Contamination of food products.

1.10.1 Sources of contamination and spread of microorganisms in food processing environments.

Contamination of food products may arise from four main sources, namely, the

constituent raw material (see Section 1.7), people (and other animals/pests), air and surfaces (Holah *et al.*, 1994).

Because *Listeria* are ubiquitous in the natural environment and on raw materials its presence in the food processing environment is almost inevitable. Cox *et al.* (1989) isolated *Listeria* spp. from 17 food factories. The organisms were present in 15-53% of all samples taken. Interestingly a trend emerged that showed that *Listeria* spp. were isolated with decreasing frequency in the order, drains, stagnant water, floors, residues and equipment surfaces indicating that the organism is present primarily in wet areas of food plants. Almost all untreated effluent samples were positive, and it was noted that treatment of these effluents within the factory precincts led to aerosol formation which could conceivably spread these organisms throughout the processing environment. Therefore, the air may act as both a source of contamination, i.e. from outside the processing area and as a transport medium e.g. moving contamination from non-product to product contact surfaces.

1.10.2 Bacterial attachment to product contact surfaces as a potential route for the contamination of food.

Food contact surfaces are subject to bacterial adherence and colonization (Gilmour, Wilson & Fraser, 1993). Frequently the term biofilm has been used to describe attached microorganisms in the food processing environment (Lewis & Gilmour, 1987; Holah & Kearney, 1992, Carpentier & Cerf, 1993; Holah *et al.*, 1994; Hood & Zottola, 1995). However, as will be described in Section 1.12, it is generally accepted that a distinction can be made between bacteria simply adhering to surfaces and those which have colonised it and grown to form a biofilm. Since the time dimension for biofilm formation in the food processing industries is relatively short, the term biofilm is, therefore, slightly misleading and is more associated with the attachment of microorganisms to surfaces rather than the development of thick biological films (Holah *et al.*, 1994).

Few reports have documented the levels of microorganisms found on surfaces in food plants. However, a unique study by Holah, Betts & Thorpe (1989) placed

stainless steel coupons in areas adjacent to food flow in plants processing baked beans, egg glaze, fish and buttermilk. After exposure to the plant environment, the coupons were removed and examined using epifluorescence microscopy. Bacterial levels in the range 3.35 x 10^3 cells/cm^2 (fish) to >4.3 x 10^7 cells/cm^2 (baked beans) were detected within 16 h of exposure.

Bouman, Driessen & Schmidt (1981) observed that one heat-resistant microorganism, *Staphylococcus thermophilus* could adhere to the pasteurised milk section of a pasteuriser, inoculating the milk at a rate of 10^6 cells ml^{-1}. It has since been noted that this type of milk contamination can be avoided by increasing the frequency of cleaning and disinfection operations (Carpentier & Cerf, 1993). Although such high levels of non-pathogenic or non-spoilage microorganisms may be tolerated, even the presence of pathogens such as *L. monocytogenes* and *Salmonella* spp. is generally not acceptable.

SEM studies have shown that *L. monocytogenes* may adhere to surfaces commonly found in the food industry. Herald & Zottolla (1988) demonstrated that *L. monocytogenes* would firmly attach to inert stainless steel surfaces at a range of pH values and temperatures. Lee & Frank (1991a) observed *L. monocytogenes* microcolony populations of 1.0 x 10^5 cells cm^{-2} on stainless steel following an 8 d incubation period at 21°C. Surfaces had been prepared by incubating steel slides in a 1:15 dilution of TSB inoculated with *L. monocytogenes* cells; slides were rinsed every 2 d and transferred into sterile medium up to 8 d. Similarly, Mafu *et al.* (1990), demonstrated that *L. monocytogenes* Scott A could attach to stainless steel, glass, polypropylene and rubber surfaces after contact times as short as 20 min or 1 h at 20 and 4°C respectively. Interestingly, bacteria were frequently observed entrapped in surface irregularities including scratches (glass), grooves and crevices (steel), holes (polypropylene) and deep holes and crevices (rubber). However, because no quantitative estimate of adhesion levels was obtained, no correlation was demonstrated between the surface irregularities of material and the ability of the *L. monocytogenes* to attach to that particular surface.

L. monocytogenes has been shown to persist on the rubber fingers of poultry pluckers and the trolleys that carry the carcasses, after cleaning (Toquin, Lahellec &

Collin, 1991). Notermans, Dormans & Mead (1991) showed that after several weeks of operation the whole surface of the rubber plucker finger was pitted thus trapping and protecting microorganisms from the routine cleaning and disinfection process.

The fact that *L. monocytogenes* can adhere to the surfaces described above illustrates the potential for attachment to a host of other food-contact and non-food contact surfaces (Mafu *et al.*, 1990).

1.11 Control of *L. monocytogenes* in food and food processing environments.

Provided that the process environment and the production equipment have been well designed, cleaning (Section 1.11.1) and disinfection (Section 1.11.2), or referred to together as sanitation, provide the major day to day control of the 'surface' route of food production contamination (Holah *et al.*, 1994). It is, however, well established that bacteria attached to surfaces are more resistant to antimicrobial agents than those grown in planktonic culture (Bolton *et al.*, 1988; Mustapha & Liewen, 1989; Anwar & Costerton, 1990; Frank & Kofi, 1990; Lee & Frank, 1991a; Wright, Ruseska & Costerton, 1991; Dhaliwal, Cordier & Cox, 1992) (see Section 1.11.2 for studies on the resistance of *L. monocytogenes* to disinfectants). For this reason, food manufacturers often rely on secondary barriers such as food additives to minimise the growth of contaminating microorganisms in complex food products (Section 1.11.3).

As a preliminary investigation to this research, two food manufacturers X and Y were visited during July 1992. Both manufacturers specialised in the production of individual salad and mixed salad products. Information regarding the control of *Listeria* in these two manufacturing environments will be referred to in the following sections.

1.11.1 Manual procedures to eliminate *Listeria* from food contact surfaces and raw materials.

Cleaning may be used to reduce contaminating microorganisms present on both processing surfaces and raw materials. Although cleaning procedures have been shown to reduce bacterial numbers on processing surfaces by up to 6 $\log_{10}$ orders (Schmidt & Cremmling, 1981), studies have shown that the efficiency of such procedures may

decrease if the surface is repeatedly damaged, abraded or has limited accessibility. Holah & Thorpe (1990) showed that no significant differences in the cleanability of stainless steel, enamelled steel, mineral resin, high density nylon, PVC, polypropylene and polycarbonate materials existed when the surfaces were new. However, when subject to abrasion or impact damage, differences in cleanability of 2 $\log_{10}$ orders were observed between materials. Differential cleanability was related to surface topography such that the greater the degree of surface change, the greater the retention of microorganisms. The authors suggested that this could be due to an increase in bacterial attachment sites (for a given surface area), stronger bacterial adhesion due to increased bacterial/material surface area interfaces and enhanced protection from cleaning shear forces. Consequently, although cleaning procedures should be vigorous enough to remove adherent microorganisms, they should not be abrasive.

Procedures for cleaning salad vegetables include washing, peeling and removing outer leaves (Manufacturers X & Y). Washing procedures can reduce microbial populations quite effectively if done properly. However, such procedures can just as easily spread contaminants over produce, thus intensifying the potential for microbial contamination (Brackett, 1992). For example, if the same water is recycled, it will likely accumulate debris and increase microbial populations. Washing therefore tends to clean produce early in a shift but contaminates produce late in a shift (Brackett, 1992). Additionally, washing procedures should be vigorous enough to remove dirt and debris yet should not damage the texture or composition of the vegetables (Fernández-Astorga *et al.*, 1995). Peeling and removing outer leaves can obviously be very effective in eliminating surface microorganisms. However, peeling and or cutting obviously exposes nutritious internal tissue fluids to microorganisms which may accelerate microbial growth (see Section 1.7.2). Removing outer leaves is of limited use in cases where vegetables have split or been damaged allowing microorganisms to reside on internal leaf layer which are not cleaned (Manufacturers X & Y). Thus, the efficiency of cleaning procedures (for both raw material and processing surfaces) is dependent on the accessibility of the surface to be cleaned.

Although the majority of microbial contamination is usually removed by the

cleaning phase of the sanitation programme (Holah *et al.*, 1994), for many food processing operations there are likely to be sufficient viable microorganisms remaining on the surface to warrant the application of a chemical disinfectant.

1.11.2 Chemical disinfection.

Although there are a range of chemical treatments currently available for the disinfection of the manufacturing environment, the use of chemicals on raw salad vegetables is strictly regulated. Numerous studies (detailed below) have examined the efficiency of chemical sanitizers on the inactivation of *L. monocytogenes* cells both in suspension and when adhered to inert or organic surfaces. It should, be noted that the methodology in these studies has varied considerably making direct comparison difficult. For example, biofilms have been developed over 1 h to 14 d incubation periods, using minimal and complex growth media and at a range of incubation temperatures. In addition, methods for biofilm enumeration have varied significantly using techniques such as scraping, swabbing, vortexing with glass beads and epifluorescence. Each technique has its own advantages and disadvantages.

1.11.2.i Hypochlorite solutions.

Hypochlorites (mainly of 2 types; sodium- and calcium hypochlorite) are powerful germicides effective against a wide spectrum of organisms (El-Kest & Marth, 1988ab). They are non-poisonous to man at used concentrations (typically 50-100 ppm) and do not leave a poisonous residue. They are colourless, easy to handle, and economic. For these reasons, chlorine compounds are widely used for disinfection in the food and dairy industry and are one of the only chemical treatments approved for the washing of salad vegetables. Various environmental factors alone or in combination affect the germicidal activity of chlorine. These factors include pH, temperature, chlorine concentration, the presence of organic matter (particularly protein) and hardness of water (El-Kest & Marth, 1988a).

Although chlorine is very effective in killing *L. monocytogenes* populations whilst the cells are in suspension (Brackett, 1987), adherent cells have shown an

increased resistance to this sanitizer (Brackett, 1987; Lee & Frank, 1991a). Brackett (1987) demonstrated that a 50 ppm concentration of hypochlorite would kill a 10^8 cfu ml^{-1} population of *L. monocytogenes* in phosphate buffer following only a 20 s incubation period. However, Brussels sprouts containing approximately 10^6 cfu *L. monocytogenes* per gram showed only a 2 $\log_{10}$ cfu g^{-1} (99%) decrease in population after being dipped for 10 s in a 200 ppm solution of chlorine. Moreover, water was almost as effective as chlorinated water; a 1 $\log_{10}$ (90%) reductions being obtained simple by dipping the Brussels sprouts in water. Brackett concluded that although hypochlorite may be of value in removing *L. monocytogenes* from water supplies, it did not appear to be of use in removing the organism from contaminated vegetables.

Beuchat & Brackett (1990a) showed that pre-chlorine treatment (washing of lettuce with 200 to 250 μg ml^{-1} free chlorine before inoculation) did not significantly reduce the survival or growth of *L. monocytogenes* on Iceberg lettuce stored at 5 or 10°C. Although populations of *Listeria* on chlorine washed lettuce at 0 d were lower than control samples, by 15 d after inoculation cell populations were not significantly different reaching 10^8-10^9 cfu g^{-1}. It was suggested that some chlorine may have remained on the lettuce and had an initial lethal or sub-lethal effect on the cells subsequently applied.

Similarly, Beuchat & Brackett (1991) also showed that rates of death and growth of *L. monocytogenes* inoculated onto raw whole and into chopped tomatoes were not influenced by prior treatment of tomatoes with chlorine (210 to 280 μg ml^{-1}).

Lee & Frank (1991a) compared the resistance to hypochlorite of adherent single cell and microcolony *L. monocytogenes* populations on stainless steel. Interestingly, 8 d microcolony populations were up to 100 times more resistant to 200 ppm chlorine (30 s exposure) than 4 h single cell populations of the same order (1 x 10^5 cells cm^2). In contradiction to this work was the work of Mustafa & Liewen (1989) who showed that an older adherent *L. monocytogenes* culture (24 h of incubation) was less resistant to chlorine than a newly-attached culture (1 h of incubation). However, the longer incubation times (8 d vs 24 h), use of low nutrient medium and increased efficiency of recovery techniques (scraping, swabbing and rinsing vs vortexing) by Lee & Frank

(1991a) may explain these differences.

Approaches to the use of hypochlorites in the processing industry may vary between different manufacturers. Manufacturer X used large volumes of hypochlorite to wash floors and contact surfaces during normal working hours. This approach reduced the build up of debris and consequently minimised nutrient availability and thick biofilm development. However, this approach also resulted in significant aerosol production, movement of debris (including vegetable material) and consequently the potential for spread of microorganisms throughout the entire manufacturing environment. Manufacturer Y minimised the use of hypochlorite during regular working hours and instead relied upon a thorough sanitation programme at the end of the working day. Obviously this approach also has its advantages and disadvantages. However, given that adhering *L. monocytogenes* populations are quite resistant to hypochlorite, it is generally believed that protocols which lead to the spread of the microorganism around the processing environment should be minimised as far as possible.

1.11.2.ii Quaternary ammonium compounds (QAC), listeriaphages, additional sanitizers and detergents.

Using suspension tests, van de Weyer, Devleeschouwer & Dony (1993) demonstrated from, <3.0 to 7.2 $\log_{10}$ reductions in viable *L. monocytogenes* populations using nine different disinfectants. Disinfectants included quaternary ammonium compounds (QAC), phenolic compounds, alcohols, surface-active agents, aldehydes and dischlorine tablets. Efficiency of all disinfectants was reduced by the presence of organic matter (consisting of 3% bovine albumin and 1.5% yeast extract), but was generally not strain dependent.

The susceptibility of surface-adherent single cells and microcolonies of *L. monocytogenes* on glass microscope slides to the QAC benzalkonium chloride (100 to 800 ppm), anionic acid (200 to 400 ppm) sanitizer was investigated by Frank & Kofi (1990). Planktonic cells were reduced to undetectable levels within 30 s exposure to the lowest sanitizer concentrations. Adhering microcolony cells decreased by only 2-3 $\log_{10}$

cycles immediately after exposure, while adhering single cells showed an initial 3-5 $\log_{10}$ decrease. Interestingly, exposure of the remaining adherent cells to the highest sanitizer concentration reduced viable numbers to undetectable levels after 20 min of exposure for adherent microcolonies and 12 to 16 min for single cells. Additionally, removing adherent cells from the surface increased their susceptibility to nearer that of planktonic cells.

Viability of planktonic *L. monocytogenes, Pseudomonas fluorescens* and *Yersinia enterocolitica* populations have been shown to decrease by 5 $\log_{10}$ cycles (99.999%) in suspension tests with QAC, iodophor, acidic anionic, and fatty acid sanitizers (Mosteller & Bishop, 1993). Using very effective epifluorescent, impedance and standard microbiological techniques, these authors demonstrated that all of these sanitizers failed to reduce the viability of *L. monocytogenes* populations by 3 $\log_{10}$ orders (99.9% reduction) when adhered to both Teflon and Buna-N rubber surfaces, and in only certain combinations were *P. fluorescens* and *Y. enterocolitica* populations reduced by the desired 3 $\log_{10}$ orders. Sanitizer resistance was attributed to the presence of a glycocalyx on adherent cells which is lacking in planktonic cells.

Krisinski, Brown & Marchisello (1992) studied the efficiency of a variety of chemical cleaning and disinfectant compounds in the removal and/or inactivation of adherent *L. monocytogenes* on stainless steel and plastic conveyor belt materials. Adhesion to all surfaces was found to increase to maximum levels (1×10^4 cells cm^2) following 24 h incubation of surfaces in inoculated TSB at 25°C. Results indicated that resistance to disinfectants including sodium hypochlorite, iodophor, acidic and neutral QAC, a mixed halogen, alkyl sulphonate, peracetic acid and chlorine dioxide depended largely on the surface studied, resistance being greatest on polystyrene/polyurethane surfaces followed by polyester and stainless steel. On stainless steel, all of the disinfectants with the exception of chlorine and iodophor inactivated all detectable adherent organisms within 10 min of exposure. Conversely in the case of polystyrene/polyurethane, none of the biocides were effective. In general, the most effective disinfectants were acidic QAC, peracetic acid and chlorine dioxide.

The effect of detergents on the removal of adherent cells was also dependent on

surface type (Krisinski *et al.*, 1992). Whilst all detergents tested were effective in removing adherent cells from stainless steel, on polyester and polyurethane surfaces most of the adherent cells remained even at high usage levels of detergent (e.g. 10% chlorinated alkaline solutions). Interestingly, complete biofilm removal and/or inactivation was obtained in many cases when the surface was first cleaned prior to exposure to a disinfectant. These data, therefore, support conventional wisdom that cleaning must precede disinfection in order to remove and inactivate microorganisms.

Listeriaphages, have been shown to be as effective as a 20 ppm concentration of QAC for disinfecting stainless steel and polypropylene surfaces contaminated with *L. monocytogenes* cells (Roy *et al.*, 1993). The authors suggested that listeriaphages may provide a specific means for decontamination of product contact surfaces and possibly as food additives. Although the listeriaphages used were resistant to 50 ppm QAC, their efficiency in a processing environment is dependent on their resistance to additional disinfectants used for the control of microorganisms other than *L. monocytogenes*.

Evidence has suggested that sub-optimal concentrations of sanitizing agents may induce injury of planktonic *Listeria* cells rather than cell death (Sallam & Donnelly, 1992). Using enrichment techniques, these authors demonstrated the ability of *Listeria* to repair sublethal damage after exposure to <25 ppm concentrations of four commonly used sanitizers. This research emphasises the importance of using lethal concentrations of sanitizer for optimal exposure times. The misuse of sanitizers may result in organisms not being detected in the processing environment (unless enrichment is used) and consequent contamination of food products where sublethally injured cells may recover and multiply to potentially lethal levels.

1.11.3 Irradiation.

Some food scientists have proposed irradiation as a process to eliminate microorganisms on fruit and vegetables (Kader, 1986). However, it is known that under low doses of irradiation leafy vegetables can change colour and some stone fruits experience tissue softening (Patterson, 1989). Although the potential health benefits

from irradiation technology are considerable, consumer attitudes and concerns regarding safety have generally restricted its use. However, recent studies in the U.S. indicate that such concerns have decreased considerably worldwide in the past 10 years, with consumers now being more worried about pesticide residues and microbiological contamination (Bruhn, 1995). Thus, irradiation may have increasing applications in future years.

1.11.4 Food additives.

The difficulty in eliminating *L. monocytogenes* totally from raw ingredients often results in the need for further hurdles to inhibit/eliminate the growth/survival of the bacterium in complex food products including ready-to-use vegetables. A wide variety of food additives are currently available, however, their efficiency is often dependent on a fine balance between the food type, additive concentration, salt, temperature, pH and type of acidulent used.

1.11.4.i Acids and salts.

Food acidulents frequently serve as inhibitors of unwanted microbial growth (Conner *et al.*, 1990). The most frequently used acids in the food industry are lactic, acetic (vinegar), citric and tartaric acid. These acids are frequently incorporated into food products of high moisture content such as coleslaw or yoghurt. However, as described below, the effect of acids on *L. monocytogenes* growth in such foods is dependent on pH, salt levels and temperature (Conner *et al.*, 1990).

L. monocytogenes is quite tolerant to sodium chloride. Sorrells & Enigl (1990) showed it could grow in TSB containing 10% NaCl at 35°C and 12% NaCl at 25 and 10°C. The maximum NaCl concentration for growth changed when NaCl and pH, in combination with different acidulents and temperature, were tested. Shahamat, Seaman & Woodbine (1980), showed that nitrite inhibited growth of *L. monocytogenes* only with $>$ 3.0% NaCl and a pH value at or below 5.5. Junttila *et al.* (1989) showed that growth and multiplication of *L. monocytogenes* in fermented sausages (with final pH levels between 4.3 and 4.6) was prevented by 3% NaCl and 120-150 ppm $NaNO_2$,

however, survival of the bacterium was not affected by these currently permitted salt concentrations or even those higher.

Conner, Brackett & Beuchat (1986) demonstrated that growth of *L. monocytogenes* in unclarified cabbage juice was also dependent on a fine balance between storage temperature, pH and the concentration of NaCl added to the medium. In cabbage juice containing $\leq$ 1.5% NaCl, *L. monocytogenes* multiplied from 10^4 to 10^9 cfu ml^{-1} within 8 d when incubated at 30°C, however the population decreased rapidly to undetectable levels by 25 days. In contrast when incubated at 5°C, no increases were observed but the initial cell populations remained stable over a 70 d incubation period. In addition, *Listeria* cells were more resistant to increasing levels of NaCl at 5°C than at 30°C. At 2.5% NaCl concentrations, viable populations declined from 0 d to non-detectable levels within 15 days at 30°C, whereas at 5°C populations remained stable over a 42 day incubation period. By lowering the initial pH of the cabbage juice with lactic acid, initial cell populations decreased at faster rates than that seen at higher pH. However, the rates of inactivation were lower at 5°C than at 30°C indicating that low temperature may afford some protection to *L. monocytogenes* against the stress effect of both low pH and high NaCl concentration.

Interestingly, Conner *et al.* (1986) also noted that heavy growth (10^8-10^9 cfu ml^{-1}) of *L. monocytogenes* in cabbage juice with and without added NaCl resulted in substantial decreases in cabbage juice pH. At 8 d, the pH of samples in which heavy growth was observed had decreased from 5.6 to 4.3. No pH change was observed in samples in which the population did not increase indicating that growth of *L. monocytogenes* caused the reduction in pH. This was attributed to the bacterium fermenting natural sugars such as sucrose present in the cabbage juice producing titratable lactic acid when the population of cells in the medium exceeded 10^8 cells ml^{-1}.

Additional studies by Beuchat *et al.* (1986) examined the thermal inactivation of *L. monocytogenes* in clarified cabbage juice at a range of pH levels. These authors demonstrated that as the treatment temperature increased, the rate of inactivation also increased, and occurred at a faster rate as the initial pH of the medium was decreased. At 56°C and pH 4.6 a D-value of 2.04 min was calculated compared to 4.74 min at pH

5.6. Hence, it was concluded that fermented vegetables which receive minimal heat processing before marketing pose little threat to public health. The highest threat to public health, therefore, appears to be in low-acid foods which are held at refrigerated temperatures prior to consumption. As indicated by Conner *et al.* (1986), *L. monocytogenes* is most likely unable to tolerate the environment of acid foods and high-acid foods for extended periods of time. Interestingly, manufacturer Y reported that ready-to-eat salad containing fruits e.g. 'Florida' salad, although high in sugar, very infrequently contained *Listeria*. This was attributed to the naturally high acidity of these salads.

1.11.4.ii Natural antimicrobial systems.

Intrinsic natural antimicrobial systems have been suggested as a potential means of food preservation (Banks, Board & Sparks, 1986). These systems which may include the addition to foods of biological control agents (e.g. lactic acid bacteria), plant and other natural extracts are particularly attractive because they are preferred by the consumer to conventional less natural systems.

a) Lactic acid bacteria.

It is well recognized that lactic-acid bacteria produce inhibitory compounds, such as lactic acid, bacteriocins (see part b below), antibiotics or bacteriocin like substances (Klaenhammer, 1988). Wilson (1994) showed that *Lactobacillus plantarum* SK1 isolated from saukraut had anti-listerial activity against *L. monocytogenes* both *in vitro* and when inoculated onto Dutch White cabbage leaf discs. Activity was increased *in planta* when the ratio of antagonist to *L. monocytogenes* was increased. The author concluded that the anti-listerial activity of this strain was most probably due to the production of lactic acid although the production of other antimicrobial compounds was not ruled out.

Vescovo *et al.* (1995) followed the inhibitory effect of selected lactic acid bacteria on the growth dynamics of microflora on ready-to-use vegetables during refrigerated storage. *Lactobacillus* and *Pediococcus* strains were able to colonize the vegetables and dominate contaminating micro-organisms. This was correlated with a

decrease in pH from approximately 5.8 to values ranging from 5.00 to 5.29 depending on the vegetable type. It was, however, noted that the inhibitory effect of lactic acid bacteria was greater in mixed salads with a lower initial microflora load, an observation that confirms the necessity of more strict hygienic practices during processing of vegetables. Experiments are still in progress to elucidate the nature of the antimicrobial activity of these isolates which have now also shown inhibitory action against *L. monocytogenes*, *Aeromonas hydrophila*, *Salmonella typhimurium* and *Staphylococcus aureus* on MRS agar, in salads and in juice prepared from vegetable salads (Vescovo *et al.*, 1996).

b) Bacteriocins.

The potential use of bacteriocins in food preservation is well documented (Kim, 1993). The most thoroughly studied bacteriocin is nisin (Mahadeeo & Tatini, 1994). It is a peptide produced by certain strains of *Lactococcus lactis* subsp. *lactis* (Daba *et al.*, 1991). Nisin has been approved as a food additive in at least 46 countries particularly for inhibiting *Clostridium* spp. in cheese and canned foods (Harris, Fleming & Klaenhammer, 1992). The sensitivity of *L. monocytogenes* to nisin has been demonstrated by several studies in meat and cottage cheese (Chung, Dickson & Crouse, 1989; Benkerroum & Sandine, 1988). Recently, Mahadeeo & Tatini (1994) demonstrated that although nisin was very effective at killing *Listeria* suspended in scald water, its effect was significantly reduced when the cells were attached to turkey skin. It has been suggested that a combination of bacteriocins (e.g nisin and carnocin 54) in food may improve bactericidal efficiency (Schillinger, Becker & Holzapfel, 1995).

c) Plant extracts.

Chinese medicinal plant extracts have been shown to have anti-listerial activity both *in vitro* and in cabbage juice (Chung, Thomasson & Wu-Yan, 1990). Although it was found that the addition of protein (in the form 1% peptone) to the cabbage juice reduced the anti-listerial effect of the one plant extract studied, it was believed that the effective use of such extracts in low protein foods, such as salad vegetables, was

possible. Chung *et al.* (1993) also showed that other plant extracts including tannic acid (a tannin frequently found in strawberries, grapes and other plant products) and propyl gallate inhibited the growth of *L. monocytogenes* in cabbage juice. Aureli, Costantini & Zolea (1992) showed that of 32 extracted plant essential oils, five (cinnamon, clove, origanum, pimento, and thyme) had antilisterial activity *in vitro*. Tassou, Drosinos & Nychas (1995) suggested that essential oil extracted from mint (*Mentha piperita*) is a promising food preservation additive, its antilisterial activity was increased with high extract concentration, low food pH, low salt concentration, low storage temperature but was dependent on food composition.

Beuchat, Bracket & Doyle (1994) commented on the potential for carrot extracts as secondary barriers of *L. monocytogenes* growth in foods (see Section 1.8.5 for effect of carrot extracts on *Listeria*). Beuchat & Doyle (1995) demonstrated that although treatment of lettuce with carrot juice retarded the growth of *L. monocytogenes* at 12°C it was without effect at 20°C. Additionally carrot juice (10% concentration) was ineffective in controlling the growth of *L. monocytogenes* in frankfurter homogenates, regardless of the incubation temperature.

1.11.5 Storage of food.

Modified atmospheres are frequently used to extend the shelf life of food products. Berrang *et al.* (1989), showed that gas atmospheres (3-10% CO_2) did not affect the growth rates of *L. monocytogenes* on either asparagus, cauliflower, or broccoli samples. The population of *L. monocytogenes* on asparagus stored at 4°C under modified atmosphere was significantly higher after 21 d compared with samples stored under air for 14 d. Alarmingly, both storage times were end points with regard to consumer acceptability. Thus, fresh asparagus with an extended shelf life could present a greater microbial hazard than an unpacked sample. Since this was not the case for cauliflower or broccoli, the authors concluded that the safety hazard of modified atmosphere packaging should be considered separately for different vegetables (Berrang *et al.*, 1989).

Similarly, Kallander *et al.* (1991) showed that modified atmosphere (70% CO_2,

30% N_2) did not have any adverse affect on the survival of *L. monocytogenes* in shredded cabbage at 5 or 25°C. Furthermore, at low temperatures, by extending the shelf life of the cabbage, modified atmosphere packaging actually increased the duration for which the cells could survive and possibly multiply. Beuchat & Brackett (1990a) also showed that modified atmosphere packaging (3% O_2, 97% N_2) did not affect the growth characteristics of *L. monocytogenes* on Iceberg lettuce over 15 d at either 5 or 10°C when compared to control samples.

1.12 Bacterial adhesion and biofilm formation.

The ability of *L. monocytogenes* to adhere to raw materials, such as leaf vegetables or product contact surfaces, presents a significant route for contamination of minimally processed food products. Understanding the mechanisms of *L. monocytogenes* adhesion to such surfaces would represent a significant step in the development of techniques for their removal from the food supply.

The term biofilm is frequently used to describe adhering bacteria on surfaces. However, as will become clear in future sections adhesion is also commonly recognised as only one of many stages in the development of biofilms. Differentiating between bacterial adhesion and subsequent biofilm formation has led to much confusion in the scientific literature and consequently many definitions have been given to biofilms. Characklis (1989) defined biofilms as consisting of cells immobilised on a substratum often embedded in a matrix of microbially produced organic polymer. Carpentier & Cerf (1993) defined a biofilm as a community of microbes embedded in an organic polymer matrix, adhered to a surface. The latter definition, which includes the term community, indicates that biofilms may contain a variety of different microorganisms. For example, Haack & McFeters (1982) used the term biofilm to describe a community of filamentous algae, diatoms and bacteria embedded in an extracellular polysaccharide (EPS) matrix which they isolated from an alpine stream. Additionally, describing the microbial populations within biofilms as a functional consortia (Gilbert, 1995) indicates that most recognised biofilms perform activities and processes at different rates than those in other environments. Furthermore, this suggests that biofilms are ecological

niches which perform functions not found, or rarely found, elsewhere.

To fully appreciate the implications of bacterial adhesion it is important to give some examples of the interfaces (Section 1.12.1) and environments (Section 1.12.2) in which adhesion and subsequent biofilm formation may occur. Additionally, the benefits that life on a surface or in a biofilm offers to the microorganism (Section 1.12.3) and the subsequent problems such biofilms cause (Section 1.12.4) should be considered to fully appreciate the importance of the initial adhesion event to both the microorganism and man.

1.12.1 Types of interfaces at which biofilms occur.

The term biofilm is usually used to describe microorganisms associated with solid substrata. Biofilms have been found on almost all submerged surfaces within the natural environment (Lappin-Scott, Jass & Costerton, 1993) as well as surfaces which are periodically immersed (Madsen & Ghiorse, 1993) or are located in relatively humid environments (Noble, 1981).

Additionally, other interfaces such as air-liquid and liquid-liquid, also attract and associate distinct communities of microorganisms, the former of which are termed neustonic biofilms (Marshall, 1976; Hermansson & Dahlbäck, 1983). Neustonic biofilms can form flocks with respects to activated sludge or mats in large lakes (Cohen & Rosenberg, 1989). Interestingly, the phenomena which attract and cause microorganisms to flocculate at the air-liquid interface are essentially the same as those which determine adhesion to solid surfaces (Daniels, 1980). The main difference between solid and air, as an interface with water, concerns the variety and surface heterogenicity of solid substrata.

Solid/liquid interfaces probably represent the most frequently encountered sites of biofilm formation (Gilbert & Alison, 1993). They are also of greatest importance in medical, industrial and environmental sectors (Wimpenny, Kinniment, & Scourfield, 1993). For these reasons the following sections will be concerned only with this interface.

1.12.2 Environments in which biofilms may develop.

Biofilms may occur in fresh (Haack & McFeters, 1982), salt (Pedersen, 1982), estuarine (Jeffrey & Paul, 1986), groundwater (Mills *et al.*, 1994), drinking water (Ridgway, Means & Olsen, 1981) and swimming pools (McCormick & Cairns, 1990). The physiology, metabolism and organisation of biofilms is greatly dependent on the nature of the surfaces within these environments. Typical surfaces in these environments on which biofilms occur include stones (Geesey, Mutch & Costerton, 1978), sediment (Williams, 1984), marine craft and structures (Cooksley & Wigglesworth-Cooksey, 1992), soil particles (Laanbroek & Geerlings, 1983), piping (Rogers *et al.*, 1994), vegetation (Dazzo, 1980) animal life (Cahill, 1990) in addition to the host of surfaces commonly found in food manufacturing and processing environments (Gilmour *et al.*, 1993; Holah *et al.*, 1994).

1.12.3 Advantages of life as a biofilm.

The ability of microorganisms to adhere to and form biofilms on surfaces conveys upon them many advantages relative to life in the bulk phase. For example, researchers have demonstrated that microorganisms may display 10-3000 times more resistance to biocides than planktonic cells (Flemming, 1991; Dhaliwal *et al.*, 1992), a phenomenon which has also been observed for *L. monocytogenes* cells (Section 1.11.2). Adherent cells may also be more resistant to heat (Frank & Koffi, 1990) and bacteriophages (Hicks & Rowbury, 1987). Additionally, surfaces may provide nutrients/cations for microbial utilisation (Gilbert & Alison, 1993). For example, leaf tissue which has been cut or damaged may release nutrients from internal leaf cells which then accumulate at the leaf surface. Alternatively, surfaces may also adsorb organic material from the bulk phase in the form of a 'conditioning' layer which may also act as a substrate for microbial growth (Fletcher, 1991). For some developed biofilms, the polysaccharide fibres in the organic polymer matrix, which are generally negatively charged, may trap the organic and mineral molecules and particles circulating in suspension in the vicinity of the biofilm (Carpentier & Cerf, 1993). Thus, adhesion may offer microorganisms both a strategy of surviving in hostile environments and a

means of growth in nutrient depleted conditions.

1.12.4 Implications of biofilms for man.

Bacterial adhesion and biofilm formation have various interests to man, some of them positive, for example, sewage and water treatment (Eighmy, Maratea & Bishop, 1983), biotechnology (Yu & Pinder, 1992) and pollution control (Woolfaardt *et al.*, 1994), whereas others have negative implications. All are intensively studied in contexts of ecology, agriculture, medicine, engineering and technology (for biofilm reviews see Berkley *et al.*, 1980; Bitton & Marshall, 1980; Denyer, Gorman & Sussman, 1993; Quesnel, Gilbert & Handley, 1993; Ofek & Doyle, 1994; Wimpenny *et al.*, 1994, 1995; Doyle & Ofek, 1995). The common feature of all these studies is, however, the wish to understand and master adhesion whether the ultimate aim is to promote or prevent it.

Biofilms formation on surfaces may cause both direct and indirect problems for man. Indirectly, for example, biofilm growth of single-celled algae, diatoms and bacteria on a ship hull is known to entice barnacles to colonise the surface, resulting in increased fluid frictional resistance thus slowing the ship down and increasing fuel consumption (Cooksley & Wigglesworth-Cooksey, 1992). More directly, when biofilms contain sulphate-reducing or acid producing bacteria they can cause corrosion. These microorganisms create anodes and cathodes on metal surfaces; the unequal distribution of ions causes currents, resulting in a metal loss (Costerton *et al.*, 1987; Videla & Characklis, 1992). This may result in localised pitting of the surface with potentially catastrophic effects on pipelines and oil rigs (Lappin-Scott & Costerton, 1989). Biofilm growth in pipelines may also reduce the diameter of pipes sufficiently to restrict the movement of fluid, a problem of particular concern if this prevents efficient heat exchange in cooling water systems (Cloete, Brözel & Holy, 1992). Similarly, biofilms up to 500 μm thick have been observed on filtration system membranes resulting in a significant reduced permeability (Flemming, Schaule & McDonogh, 1992).

Tap water distribution networks are also prone to develop biofilms (LeChevallier, Babcock & Lee, 1987). Such biofilms may pose a significant health

threat if they contain pathogenic microorganisms such as *Legionella pneumophila*. This microorganism thrives in such low nutrient environments causing significant problems particularly when the release of biofilm cells into planktonic suspension is followed by a dissemination of the organism through cooling towers and ventilation systems such as those found in hospitals (Lee & West, 1991; Rogers *et al.*, 1994).

The occurrence of biofilms in clinical environments has received considerable attention for many years. Natural surfaces of the body support attached microorganisms which are commonly commensal and non-pathogenic but may also contain pathogenic microorganisms. A well studied example is that of plaque biofilm attached to tooth enamel which under certain circumstances may cause periodontal disease (for reviews of this subject see Rolla, Ellingsen & Herlofsen, 1991; Busscher, Cowan & van der Mei, 1992; Busscher, Quirynen & van der Mei, 1992; Quirynen & Bollen, 1995). Other examples of natural surfaces supporting biofilm formation include the skin (Noble, 1981), internal epithelial tissue (Savage, 1980), soft wound tissues (Lippincott & Lippincott, 1969) and bone joints (Marie & Costerton, 1985) (also see Doyle & Ofek [1995] for an extensive review detailing the bacterial adhesion of microbial pathogens to host tissue). The colonization of artificial surfaces used clinically, such as sutures (Olson, Ruseska & Costerton, 1988), catheters and other medical implants (Brown *et al.*, 1985; Ladd *et al.*, 1985; Christensen *et al.*, 1989; Sheretz *et al.*, 1990) and contact lenses (Gandhi *et al.*, 1993) is also a common cause of disease and source of infection (Patrick & Larkin, 1993). In spite of the stimulation of the immune response to such infection, cells within the deeper layers of multi-layered biofilms can resist both antibodies and phagocytic white blood cells. Consequently replacement of the surface is usually the only means of eliminating the biofilm (Anwar & Costerton, 1992).

As previously mentioned, the food industry is obviously not immune from the phenomenon of microbial adhesion, followed in some cases by a build up of biofilm (Holah & Kearney, 1992; Holah *et al.*, 1994) (also see Section 1.10.2). Czechowski (not dated, a & b) reported on biofilms found in dairies and breweries, on bends in pipes, rubber seals, conveyor belts, waste water pipes, floors etc. In general, all unsterilised equipment harbours microorganisms which, between two cleaning and

disinfection operations, have the time to start a colonization process (Carpentier & Cerf, 1993).

Although adhering *L. monocytogenes* cells pose far greater problems for food manufacturers than their free-floating planktonic counterparts, few workers have examined the mechanisms of *L. monocytogenes* adhesion to either inert or organic raw material surfaces. As previously mentioned an understanding of the mechanisms of *L. monocytogenes* adhesion to such surfaces would facilitate the development of more effective cleaning and disinfection strategies than those currently available. For this reason, the remainder of this chapter will concentrate on some current concepts and theories used for the study of bacterial adhesion and subsequent biofilm formation.

1.13 Stages in biofilm formation.

The formation of biofilms on a collecting surface is usually divided into a number of individual time-dependent stages. Initially a microorganism must approach a surface and get close enough to it to form an adhesive bond. This attachment is the first step in the colonisation of surfaces and precedes the process of consolidation, during which the initial weak adhesive forces are strengthened, frequently involving exopolymer formation. Finally growth and division of the microorganisms may occur to form an established biofilm (Denyer *et al.*, 1993).

Each of the above stages may themselves be divided into a number of discrete events. The ability of microorganisms to overcome each of these events generally determines to what extent a biofilm will be formed, if at all. In the following sections only the initial stages of biofilm formation, i.e. transport (Section 1.14) and adhesion/consolidation (Section 1.15) will be described as these are of greatest importance when trying to prevent or reduce surface contamination.

1.14 Transport of bacteria to the surface.

Adhesion of bacteria to solid surfaces occurs both in quiescent waters and in conditions of turbulent flow. For each of these conditions, the mechanisms of transport from the bulk liquid to the vicinity of the solid-liquid interface may differ considerably.

Bryers & Characklis (1981) showed that the rate of overall bacterial film development on surfaces in turbulent flow was increased with biomass concentration dispersed in the bulk phase. Consequently, in very dilute suspensions of bacteria (as in open ocean waters) transport is probably the rate-limiting step in the process of bacterial deposition at the surface (Characklis, 1981a).

Currents in water bodies provide the major mechanism for the transport of bacteria over large distances. Eddy diffusion in turbulent flow systems tend to disperse bacteria resulting in a uniform concentration in the bulk water phase (Characklis, 1981a), but near the surface the bacteria are transported to the region of the viscous (or boundary) layer. When the bacteria are travelling at a greater velocity than the water in the viscous layer, then a lift force directs the bacteria towards the surface where frictional drag forces slow them down (Characklis, 1981a). In fast flowing systems, turbulent downsweeps (Marshall, 1985) also direct bacteria towards the surface. At very fast flow rates, shear forces may begin to displace adherent cells (Mittleman *et al.*, 1990).

Bacteria in low shear systems may be transported by a number of mechanisms. These include: 1) Sedimentation; this is dependent on the relative size of bacteria or bacterial aggregates (Characklis, 1981b). 2) Chemotaxis; motile bacteria are capable of displaying a positive chemotactic response to certain nutrient sources (Alder, 1969) and, as such, can respond to a nutrient concentration gradient that exists at a solid-water interface. Bacteria may also exhibit negative chemotactic response where inhibitory substances, such as hydrogen ions or antibiotics, accumulate at the solid-water interface (Young & Mitchell, 1973). 3) Brownian motion; Small bacteria (less than 1 μm in diameter) exhibit a significant degree of Brownian displacement, and such motion can account for random contacts by bacteria with surfaces in quiescent conditions (Characklis, 1981b; Marshall & Blainey, 1991).

The above mechanisms provide a means for transporting bacteria from the bulk aqueous phase to the vicinity of the surface. The adhesion of bacteria to solid surfaces involves a consideration of various aspects of substratum and bacterial surface properties, colloid chemistry along with an understanding of some physiological

responses of different bacteria to the conditions existing both in the bulk liquid and at the solid-water interface.

1.15 Adhesion of bacteria to solid surfaces.

Up to now no uniform theory has been developed to explain the fundamental mechanisms of cell adhesion (Quirynen & Bollen, 1995). Moreover it would be impossible and erroneous to conclude that one single mechanism dictates the adhesive tendency of microorganisms because the situation is too complex (Ho, 1986). According to Hodd & Zottola (1995), the most generally accepted theories for the attachment of bacteria to solid surfaces are a two step process (Marshall, Stout & Mitchell, 1971) and a three step process (Busscher & Weerkamp, 1987). In the two-step model, the first step involves the bacteria being transported close enough to the surface so that they can be adsorbed onto the surface. This step is reversible, i.e. the bacteria can be removed by simple washing. Once adsorbed, the second step is time dependent and involves the production of extracellular material that anchors the bacteria to the surface.

The three step model proposed by Busscher & Weerkamp (1987) views the adhesion process in terms of distance of the bacteria from the surface. At distances of > 50 nm, only long-range forces operate and the adhesion is reversible. As the separation distance approaches 20 nm, both long-range and electrostatic interactions are operating. This step may be reversible but over time becomes irreversible. The third step occurs at distances < 15 nm where additional forces come into play such as the production of adhesive polymers that lead to irreversible attachment. In both models, the final step depends on the ability of the microorganisms to metabolise and produce adhesive material.

For the initial steps of adhesion to occur attractive forces between the cell and the substratum must outweigh any repulsive forces. Considerable conflict among researchers has arisen through efforts to explain or predict adhesion in terms of the balance between attractive and repulsive forces. One of the oldest theories for particle adhesion is the now classical DLVO or colloid stability theory developed independently by Derjaquin & Landau (1941) and Verwey & Overbek (1948). According to this theory

the interaction Gibbs energy between a particle and a macroscopic surface is considered to be determined by the sum of London van der Waals attraction forces and electrostatic forces due to overlap of electrical double layers. Marshall *et al.* (1971) was the first author to propose this theory to explain bacterial adhesion. Since then the simplified colloid chemistry approach has been refined considerably to account for the biological complexity. The Wetting theory (or surface energy approach) considers bacterial cell adhesion in terms of the cell-surface and substratum hydrophobicity, and predicts that adhesion will occur if it results in a decrease in the surface free energy of the system. Different equations have been suggested to calculate the surface free energies from contact angles between liquids and surface on the one hand and microorganisms on the other (Bellon-Fontaine *et al.*, 1990).

As pointed out by Pethica (1980), the connections between the DLVO theory and the Wetting theory have not been fully appreciated. Pethica emphasizes the problems existing with both approaches and considered that a general descriptive framework was required for biological adhesion which takes account of experimentally accessible variables and which provided a basic critique for the DLVO and wetting theories.

1.15.1 Initial adhesion.

With particular reference to the DLVO theory, Rutter & Vincent (1980) introduced the basic physico-chemical concepts involved in adhesion for rigid, spherical charged colloid particles adsorbing at a planar interface from aqueous solution. To approach the situation of microbial adhesion they then modified these ideas for the case of non-spherical, non-rigid particles in the presence of macromolecules such as extracellular polysaccharide. Both concepts were based on the fact that a colloid particle/microorganism and a surface interact with each other from a certain distance through (weak) long-range and (strong) short-range forces.

1.15.2 Long-range forces.

When colloid particles or bacteria approach a surface, they initially interact with that surface by means of two forces: van der Waals forces (the first force to become

active at distances even above 50 nm) and electrostatic forces (at closer approach) (Rutter & Vincent, 1980).

1.15.2.i van der Waals forces (G_A).

Three types of van der Waals forces have been identified: i) London dispersion; when 2 atoms approach each other up to a certain separation gap, they will attract each other due to an instantaneous induction of dipoles (relative change in the position of the electrons in relation to the neutrons). ii) Debye forces; when a molecule (which normally possesses a dipole) reacts with an atom, a dipole-induced dipole situation is created. iii) Kesson forces; when two molecules approach each other a dipole-dipole interaction appears (Quirynen & Bollen, 1995). The Gibbs (G) energy of such an interaction between two particles at a given distance is expressed by the Hamaker constant (Hamaker, 1937).

These Hamaker constants are different for different surfaces and the net Hamaker constant (A) determines the van der Waals interaction energy. If A is negative then G_A is positive, and the net effect of the van der Waals forces is a repulsion. This only occurs in a few cases when the Hamaker constants for the substratum and/or the particle are $<$ than the Hamaker constant for the bulk medium. If A is positive then G_A is negative and the net effect of the van der Waals force is attraction. This is the usual situation (Rutter & Vincent, 1980).

1.15.2.ii Electrostatic forces (G_E).

When a solid is immersed in an aqueous environment it usually acquires a surface charge, either by adsorption of ions or by ionization of surface groups. The surface charge, therefore, depends to a great extent on the composition of the aqueous phase. The charge in the bacterial cell wall originates from carboxyl, phosphate and amino groups (James, 1991). The degree of dissociation of these ionic and cationic groups is determined by the pH and the activity of the surrounding electrolyte solution. Almost all bacterial cells are negatively charged at neutral pH, because the number of carboxyl and phosphate groups is generally higher than that of amino groups (van der

Wal, 1996).

Once a surface has become charged it will attract counter (oppositely charged) ions from the surrounding aqueous phase to form a Stern layer (Fig. 1.1). This process is opposed by thermal motion of the counter ions tending to distribute them evenly throughout the aqueous phase. The effects of electrostatic attraction and thermal motion on the counter ions lead to the formation of a region next to the charged surface where the concentration of counter ions is greater than in the rest of the aqueous phase. This is often referred to as the Gouy-Chapman diffuse electric double layer (Fig. 1.1) (Fletcher, 1980).

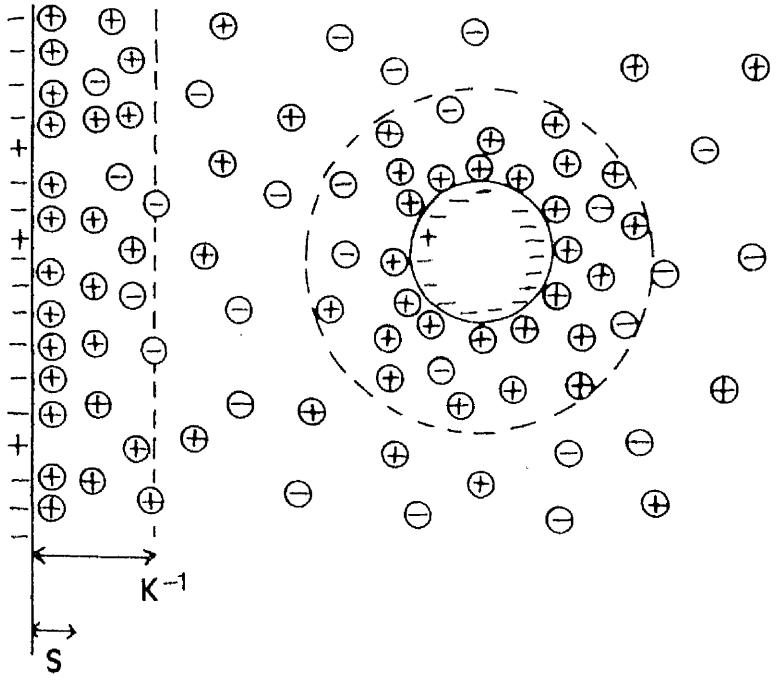
When the double layer of a particle overlaps the double layer of the surface an electrostatic interaction will be created. If both surfaces have the same charge the electrostatic interaction will be of a repulsive nature. However, if both structures have an opposite charge an attraction will occur. The energy of this electrostatic interaction is determined by the zeta potential (parameter of electrostatic charge) of the surfaces (Rutter & Vincent, 1984). The distance at which this interaction appears, depends on the thickness of the double layer, which themselves depend on the ionic charge of the surface and the ionic concentration of the suspending medium. For similarly charged surfaces in a high ionic strength solution, the double layers are small so that both surfaces can approach each other much closer before repulsive electrostatic interactions occur (Marshall *et al.*, 1971; van Loosdrecht *et al.*, 1989).

As well as ionic strength, the valency of the counter ions will also affect electrostatic interactions (van Loosdrecht *et al.*, 1987b). Direct observations (Marshall, 1971) of the attraction of *Achromobacter* R8 to glass surfaces showed that repulsion of the bacteria from the surface increased as the electrolyte concentration decreased. The concentrations at which complete repulsion occurred were $\approx 5 \times 10^{-4}$ M for NaCl and $\approx 5 \times 10^{-5}$ M for $MgSO_4$. This difference was related to the greater double layer compression in divalent systems at comparable electrolyte concentrations.

1.15.3 The DLVO theory.

The DLVO theory postulates that, above a separation distance of 1 nm the

Figure 1.1 **The electrical double layer associated with a planar surface and a spherical particle.**



S is the thickness of a layer of adsorbed counter ions referred to as the Stern layer. K^{-1} is the thickness of the diffuse double layer (Fletcher, 1980).

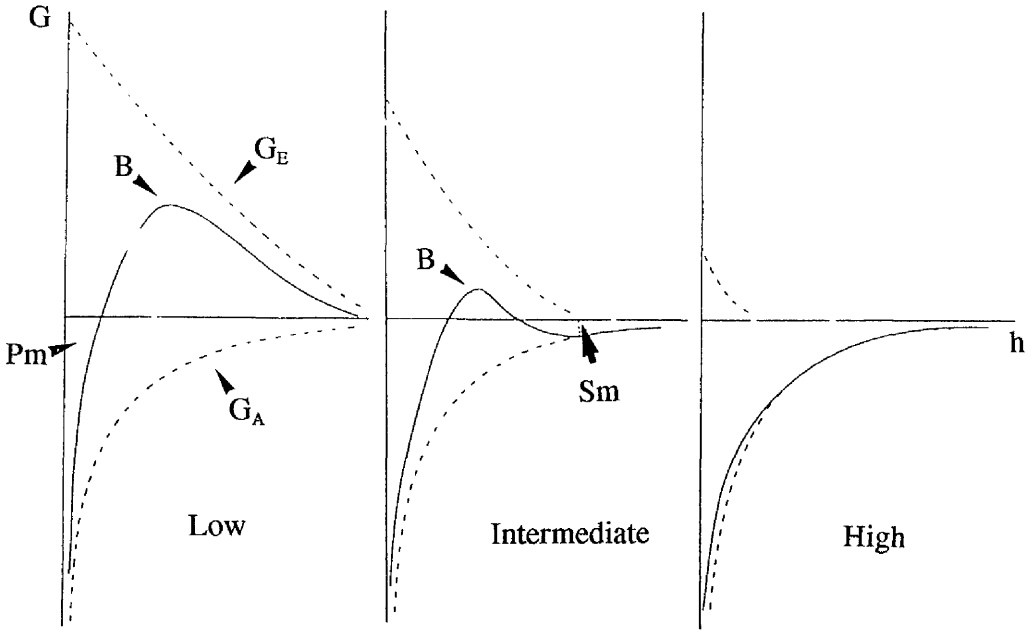
summation of van der Waals (G_A) and electrostatic (G_E) forces describes the total long-range interaction between two surfaces, also called the total Gibbs energy G_i (Verwey & Overbeek, 1948). In Fig. 1.2, the total interaction Gibbs energy is illustrated as the result of this summation ($G_i = G_A + G_E$) as a function of the separation distance (between a negatively-charged particle and a negatively-charged surface) at three electrolyte concentrations.

In Fig. 1.2.a it can be seen that at low ionic concentrations there is a large total energy barrier to overcome if the particle is to come into close contact with the surface; G_i consisting of a large positive maximum (B) (a barrier to adhesion) and a steep minimum (called the primary minimum [Pm], located at <2 nm away from the surface). Thus, at low electrolyte concentrations the particle/bacteria is effectively repelled away from the surface due to a large electrostatic double layer interaction. At high electrolyte concentrations this energy barrier is eliminated and there is a strong net attraction between the particle/bacteria and the surface (Fig. 1.2.c).

At intermediate electrolyte concentrations (Fig. 1.2.b) the free energy barrier (B) is still present but smaller due to a reduction in the range within which the repulsive electrostatic G_E forces are active. Additionally, the smaller the particle the lower the height of the energy barrier (B) so that a fraction of the particles may contain sufficient thermal energy to pass this barrier in order to reach the primary minimum where irreversible adhesion may occur. Additionally, for intermediate electrolyte concentrations a secondary minimum (Sm) is also created. The secondary minimum (considered less important than the primary minimum) is located at 5-20 nm from the surface (van Loosdrecht & Zehnder, 1990). This minimum is greater (deeper) for systems having large van der Waals attraction and large particles (van Loosdrecht *et al.*, 1989). In the secondary minimum a particle can adhere reversibly (shallow minimum) or irreversible (deep minimum) (Characklis, 1990; Quirynen & Bollen, 1995).

In those cases where the surface and the particle are of opposite charge, electrostatic interactions are attractive between the particle and the surface. The net effect is a strong attraction, decreasing with increasing bulk electrolyte concentration, but in all cases leading to irreversible adhesion (Rutter & Vincent, 1980).

Figure 1.2 **Diagrammatic representation of repulsion and attraction free energies as a function of separation distance between a charged particle (bacterium) and a macroscopic surface of the same sign.**



Interaction free energy (G) versus separation distance (h) are plotted for low, intermediate and high electrolyte concentrations. The total interaction Gibbs energy (solid line) is the product of van der Waals (G_A) and electrostatic (G_E) forces. The positive repulsion maximum (B), primary attractive minimum (Pm) and secondary attractive minimum (Sm) are indicated where appropriate.

(Adapted from Rutter & Vincent, 1980)

In nature, bacteria and surfaces are predominately negatively charged (Fletcher,1980) and microbes are considered to be large particles. Thus, a long-range interaction with a secondary and primary minimum is frequently encountered (Quirynen & Bollen, 1995). For bacteria the secondary minimum does not frequently reach large negative values (thus no strong attraction) which implies a reversible adhesion in the secondary minimum (reversible adhesion defined as a deposition to a surface in which the bacterium continues to exhibit Brownian motion and can readily be removed from the surface by mild shear or the bacterium's own motion) (Fletcher 1988; Marshall & Blainey, 1991; Korber, Lawrence & Caldwell, 1994).

1.15.4 Short-range interactions.

If a particle can reach that primary minimum (<2 nm from the surface) then interfacial water forms a barrier to further interactions (Busscher *et al.*, 1990). At this point a group of short range forces, including, chemical bonds such as covalent and hydrogen bonds, dipole interactions such as dipole-dipole (Keesom) and dipole-induced dipole (Debye) interactions, and hydrophobic interactions dominate and determines the strength of adhesion (Marshall & Blainey 1991).

1.15.4.i Hydrophobicity.

Hydrophobicity is a short range physicochemical interaction which has been considered by many workers to be important in the adhesion and proliferation of microorganisms at solid surfaces (Marshall, 1976; Rutter & Vincent, 1980; Dahlback *et al.*, 1981; Fattom & Shilo, 1983; Rosenberg & Kjelleberg, 1986; Absolom, 1988; Doyle & Rosenberg, 1990).

Hydrophobicity, although intuitively understood, remains a poorly defined term in biological literature (Doyle & Rosenberg, 1990). Physical chemists agree that hydrophobic interactions between surfaces depend in large part to the unique properties of water itself (Doyle & Rosenberg, 1990). Hydrophobic moieties when immersed in an aqueous phase are surrounded by a structural layer of water. The water molecules in such shells, which are unable to freely undergo hydrogen bonding in all directions,

are at a higher energy level than molecules in the bulk solution. When two hydrophobic moieties meet, therefore, they diminish the area of their surface in contact with water increasing the entropy of their shell. Water is thus expelled into the bulk solvent from between them, and there is a gain in thermodynamic stability of the system (Magnusson, 1980). Hydrophobic groups thus combine with each other whilst expelling water, forming hydrophobic bonds.

An important role that Busscher & Weerkamp (1987) gave to hydrophobicity is its capacity to dehydrate the junction between a bacterial cell and the substratum. With the removal of the water film, direct contact can be made between the two surfaces, via surface structures, and irreversible bonds may be established.

When considering such hydrophobic interactions in bacterial adhesion, it is obviously important to consider the hydrophobicity of both the bacterium and the substratum.

a) Measurement of microbial hydrophobicity.

Hydrophobic domains at the cell surface are due to proteins, lipids (in rare cases, e.g. actinomycete bacteria) and lipoteichoic acids (in Gram-positive bacteria). Proteins are capable of presenting hydrophobic domains on both the bacterial cell surface and extracellular appendages by adopting particular conformation and reorientation (Mozes, 1995).

For characterising the hydrophobicity of bacteria, some methods measure a net property of the entire cell surface, others measure response of limited zones/domains (Mozes & Roxhet, 1987). Bacterial adherence to hydrocarbons (BATH) is a simple quantitative test for microbial hydrophobicity, first described by Rosenberg, Gutnick & Rosenberg (1980) and further developed in later studies (Rosenberg, 1984a; Sharon *et al.*, 1986). In this test, a hydrophobic hydrocarbon is vortexed with a washed bacterial suspension, hydrophobic cells attach to the hydrocarbon oil droplets lowering the optical density of the aqueous phase after partitioning. This drop in optical density can be measured, giving a percentage of hydrophobic cells in the original cell suspension. The technique has been applied to many microorganisms and has enabled the enrichment and

isolation of hydrophobic mutants in *Acinebacter calcoacetica* (Rosenberg & Rosenberg, 1981) *Serratia marcescens* (Rosenberg, 1984) and *Streptococcus sanguis* (Cunniffe, 1993).

Hydrophobic interaction chromotography (HIC) first used by Smyth *et al.* (1978) also measures microbial hydrophobicity through their adsorption to octyl or phenyl sepharose beads packed into small columns. Additionally, the salt aggregation test (SAT) is based on the precipitation of cells from suspension ('salting out'), has been used as a measure of the relative hydrophobicity of microorganisms (Lindhal *et al.*, 1981; Rozgonyi *et al.*, 1990).

Specific markers can be used to investigate localisation of charge and hydrophobic groups on microbial cells. Cationised ferritin is employed to probe for negative charges. Colloidal gold which is both negatively charged and hydrophobic, is used to probe for positive charges and hydrophobic domains (Handley, Hesketh & Moumena, 1991). For example, a *Streptococcus sanguis* carrying lateral tufts of fibrils of two lengths at the side of the cell was probed by cationised ferritin and colloidal gold (Handley *et al.*, 1991). The ferritin attached selectively only to the short fibrils, indicating the presence of a net negative charge (pH 6.5), whereas no ferritin attached to the cell body. The colloidal gold attached both to the short fibrils and to the ends of the long fibrils. Attachment of gold to the short fibrils was pH dependent, with an optimum at pH 3.4, indicating a net positive charge below the isoelectric point of the fibril protein. Gold attachment to the long fibrils was pH independent and therefore suggested that their tips were hydrophobic.

b) Measurement of surface hydrophobicity by contact angle.

A contact angle is the direct, quantitative measurement of the wettability of a solid substrate by a given liquid (Busscher, 1985). Contact-angle determination of cell layers (van Oss, 1978; Fletcher & Marshall, 1982) may be used to describe the hydrophobic characteristics of bacterial cell surfaces. Current developments involve deposition of a bacterial lawn on cellulose nitrate or polycarbonate membrane filters, followed by contact angle measurements with the sessile drop technique (van der Mei

et al., 1991). Such measurements can be used directly as a measure of surface hydrophobicity: the greater the contact angle with water the more hydrophobic the surface; or, by using liquids with different surface tensions, such as water and diiodomethane, surface free energy calculations may be made with the geometric mean equation (Busscher *et al.*, 1984a).

Contact angles are also used extensively to characterise the hydrophobic/hydrophilic properties of solid substrata (For Reviews see Andrade, 1985, 1988; Marmur, 1993; Shanahan, 1993; Hazlett, 1993). In addition to the sessile drop technique described above, Wilhelmy balance tensiometry (Johnson & Dettre, 1969) is a well established technique for contact angle determination on solid substrata.

The well-known Young equation (equation 1.1) describes the contact angle (Θ) on an ideal (smooth, homogenous, rigid and insoluble) solid in the form:

equation 1.1: $$\tau_{sv} = \tau_{sl} + \tau_{lv} \cos\Theta$$

where τ is the surface tension (or surface free energy) and the subscripts sv, sl, and lv refer to the solid-vapour, solid-liquid, and liquid-vapour interfaces, respectively (See Fig. 1.3).

For an 'ideal' surface that is wet by a pure liquid, Young's equation predicts one and only one thermodynamically stable contact angle. In the real world, however, the 'ideal' surface is rarely found and a range of contact angles are often measured experimentally. This range of contact angles occurs because a number of 'metastable' states, each separated by free energy barriers, exist due to the intrinsic physical and/or chemical heterogenicity of surfaces (Cahn Application Notes, not dated). Typically when a wetting front is forced to move forward on a solid surface an advancing contact angle (Θa) is observed. If that liquid front is then forced to move back on the previously wetted surface a receding contact angle (Θr) which may be quite different to Θa is also often seen. The advancing angle reflects the characteristics of the low-energy (hydrophobic) portion of the surface, while the receding angle reflects the characteristics of the high energy (hydrophilic) portion (Shanahan, 1993). The difference between Θa and Θr is termed the contact angle hysteresis.

Figure 1.3 Interfacial free energies involved with contact angle formation (Busscher, 1985).

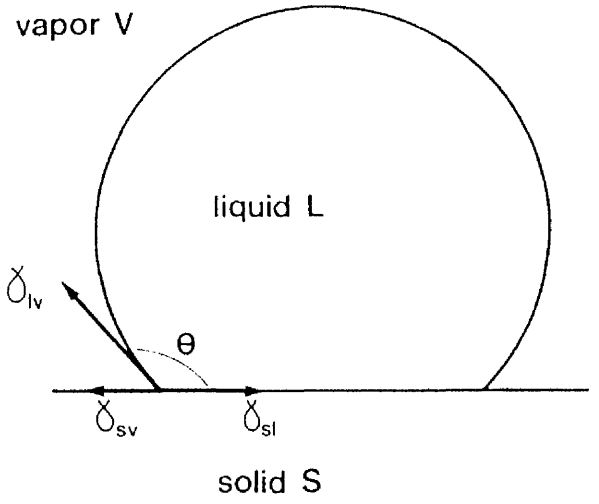
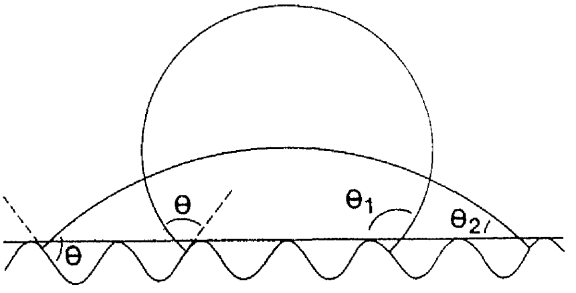
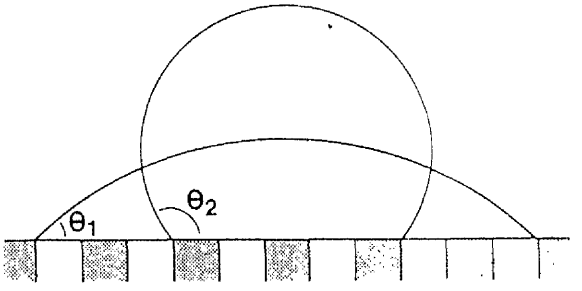


Figure 1.4 Chemical heterogenicity and surface roughness, involved in the production of contact angle hysteresis (Busscher, 1985).



influence of surface roughness on contact angles



influence of surface chemical heterogeneity on contact angles

Heterogenicity in chemical make-up of a surface (Fig.1.4), local adsorption, swelling, or dissolution of the solid by the liquid all constitute chemical sources of hysteresis (Good, 1993) whereas surface roughness is a common physical cause (Fig. 1.4) (Johnson & Dettre, 1964; Andrade, 1988). Other physical phenomena suspected to cause contact angle hysteresis are molecular orientation and solid surface deformation (Yasuda *et al.*, 1981; Busscher, 1984b; Carre *et al.*, 1987). To fully characterise any surface, and consequently predict or explain microbial adhesion it is, therefore, important to measure both advancing and receding contact angles and report the difference as the contact angle hysteresis.

1.15.5 Importance of bridging polymers in adhesion.

Bacterial surface appendages such as pili, fibrils, fimbriae, flagella extracellular polysaccharide and other proteins and polysaccharides, may act as bridging polymers, operating as a major mechanism for entry of the bacterium into the primary minimum and causing strong, short-range, irreversible and sometimes specific adhesion to the surface. These appendages may be produced either before or after initial attachment. For instance *Agrobacterium tumefaciens* has been shown to produce cellulose fibrils after adhesion to carrot cells; these fibrils anchor the cell to the surface (Matthyse, Holmes & Gurlitz, 1981). Similar observations have been made with *Rhizobium leguminosarum* (Smit, Kijne & Lugtneberg, 1987). Fimbriae produced by pathogenic bacteria have also been associated with specific adhesion mechanisms to target tissue (Duguid & Old, 1980).

In some instances bacteria appear to be held at a surface by the flagella (Meadows, 1971; Belas & Colwell, 1982). Some motile bacteria are able to overcome the attractive fores at the secondary minimum and swim away, often becoming reversibly attached at another site (Marshall *et al.*, 1971). This behaviour may be of significance in the search for nutrients at the solid-water interface.

From a study of dual occurrence of sheathed, polar flagella and lateral flagella in species of *Vibrio*, Belas & Colwell (1982) proposed a model for the possible events taking place in the adhesion of such vibrios to solid surfaces. Their model presumes that

initial reversible adhesion results from contact of the sheathed polar flagellum with the surface as suggested by Meadows (1971). After initial contact with the surface, the production of lateral flagella begins within about 3 h and may increase the forces holding the bacterium to the surface, yet still allow for movement of the bacterium on the substratum. According to this model, lateral flagella would serve as an intermediate structure between reversible adhesion, mediated by polar flagellum, and irreversible adhesion involving polymer bridging to the surface.

The extracellular polymeric substances of cells in suspension condition the surface properties of the microorganism and hence their degree of adhesion to surfaces (Shea & Williamson, 1990). ZoBell (1943) first suggested that extracellular polymers were responsible for the firm adhesion of bacteria to surfaces following microscopic observations of crystal violet-stained slides that had been immersed in seawater. Subsequently, Corpe (1970) isolated a number of strains of *Pseudomonas atlantica* from immersed surfaces, and demonstrated the production of large quantities of an acidic polysaccharide that he considered might be involved in the adhesion of these bacteria to surfaces. Marshall *et al.* (1971) reported polymer 'footprints' remaining on grid surfaces after an adherent marine pseudomonad had been sheared from the surface.

Polysaccharide conformation varies in its hydrophobicity according the environment (temperature, composition of the solvent) (Christiensen, 1989). Wrangstadh *et al.* (1990) showed that the composition of the extracellular polysaccharide (EPS) of a marine *Pseudomonas* sp. varied according to whether the bacterium was in exponential or stationary growth phase. According to Wrangstadh *et al.* (1990) the EPS formed during the exponential phase directly or indirectly favours adhesion to a hydrophobic surface (silliconized glass beads) whereas polysaccharide formed during the stationary phase have the opposite effect. With the marine *Pseudomonas* sp. studied by Christensen, Kjosbakken & Smisrod (1985) one of the extracellular polysaccharides is partly released at the end of the exponential growth phase, at the same time as bacterial adhesion to hydrophobic surfaces is increased (Fletcher & Loeb, 1979).

1.16 Bacterial colonization.

Once bacteria have adhered, the ones which produce extracellular polysaccharide reproduce and multiply within the matrix so formed. This colonisation results in the formation of what is generally recognised as a biofilm e.g. cells immobilised on a substratum often embedded in a matrix of microbially produced organic polymer (Characklis, 1989). As indicated in previous sections, factors affecting biofilm development and maturation have been extensively reviewed in the literature. For this reason and the constraints of this chapter, biofilm development will not be reviewed here.

1.17 Aims.

The overall aim of this research project was to determine the mechanisms involved in the initial adhesion of *L. monocytogenes* to leaf surfaces of salad vegetables, and more specifically to determine the influence of a range of physical and physico-chemical factors on adhesion, for example, composition of the cell-suspending medium, presence of cell surface appendages, cell surface and substratum hydrophobicity, substratum surface roughness, cell viability etc. In light of these factors the project would assess inhibitors of this binding, and determine the efficiency of washing agents in removing adherent cells.

Chapter Two

Materials and Methods

2.1 *Listeria* isolates.

Environmental isolates of *L. monocytogenes* (Murray, Webb & Swann); Pirie, *L. innocua* (Seeliger & Schoops); Seeliger, *L. ivanovii* (Seeliger *et al.*), *L. seeligeri* (Rocourt & Grimont) and *L. welshimeri* (Rocourt & Grimont) were supplied on Nutrient agar slopes (Oxoid, Unipath Ltd., Basingstoke, U.K.) by Campden Food and Drink Research Association (CFDRA), Chipping Campden, Gloucestershire, U.K. *L. monocytogenes* strains included CRA 433, CRA 1175, CRA 1177 and CRA 5246 (serotype 4b), F139 (serotype 1/2a), CRA 1100 (serotype 1/2a), CRA 1109 (serotype 1/2b) and CRA 1126 (serotype 1/2c). The serotype 4b isolate *L. monocytogenes* CRA 433 was used predominantly throughout this work.

The clinical isolate *L. monocytogenes* ATCC 23074 (serotype 4b) supplied by Nottingham University, U.K. was also used in certain aspects of the work.

2.2 Identification of *Listeria* isolates to species level.

The identity of all *Listeria* isolates supplied by the CFDRA and Nottingham University was confirmed to species level using the technique proposed by the Milk Marketing Board, Thames Ditton, U.K. A very good outline of this identification procedure was provided by Prentice & Neaves (1992). The technique was able to distinguish between the five *Listeria* species using selective agar and biochemical confirmation tests and was as follows:

2.2.1 Isolation and purification of presumptive *Listeria* isolates on selective agar.

i) Selective agar.

Listeria cultures listed in Section 2.1 were subcultured from Nutrient agar slopes, using a streak plate technique, onto Listeria Selective agar base plus Selective supplements (LSA) (CM865 + SR140, Oxoid, Unipath Ltd., Basingstoke U.K.). This

medium is also referred to as Oxford agar and was developed by Curtis *et al.* (1989) for the isolation of *Listeria* from clinical specimens containing a mixed micro-flora. The medium contains:

a) The selective inhibitory components lithium chloride, colistin sulphate, ceftotan, cycloheximide and fosfomycin to inhibit saprophytic fungi, all gram-negative bacteria and most unwanted gram-positive species, with the exception of some strains of enterococci, which grow poorly, and all *Listeria* species.

b) The indicator system aesculin and ferrous iron. *Listeria* species hydrolyse aesculin, producing a phenolic aglycon which reacts with the ferric salt in the medium to give small brown/grey colonies and blackening of the surrounding medium.

ii) Incubation of cultures.

Plates were incubated at 30°C for up to 48 h and then examined for the presence of typical *Listeria* colonies, these should be brown/grey and surrounded by a black coloration in the agar. These are presumptive *Listeria* cultures. Cultures not having this appearance were considered negative and disregarded.

2.2.2 Selection of presumptive *Listeria* cultures for identification to genus level.

Three presumptive colonies were picked from LSA for each culture and, using streak plate techniques, subcultured onto Tryptone Soy agar (TSA) (Oxoid, Unipath Ltd., Basingstoke U.K.) for separation and verification of purity. Plates were incubated at 30°C for 24 h. Following incubation, individual colonies were examined by means of the Henry Illumination system (Wood, 1969). Cultures are illuminated by white light transmitted through the colonies at an angle of 45°. Typical *Listeria* colonies are grey-blue in colour and demonstrate a fine granular texture or 'ground glass appearance'. Such colonies on TSA were used to perform all the biochemical and identification tests necessary to identify presumptive *Listeria* cultures to genus level.

2.2.3 Biochemical confirmation of *Listeria* cultures to genus level.

A number of rapid screening tests were performed to confirm presumptive cultures expressing a positive Henry illumination test on TSA as being of the genus *Listeria*. The following tests were performed on such cultures.

i) Gram stain : *Listeria* species are gram-positive.

ii) Catalase test: Performed using 3% hydrogen peroxide, the liberation of bubbles indicates a positive reaction. *Listeria* species are catalase positive.

iii) Oxidase test: Oxidase touch sticks (Oxoid, Unipath, Basingstoke, U.K.). A purple coloration of the sticks indicates a positive reaction. *Listeria* species are oxidase negative.

iv) Motility test: Performed using a wet mount hanging drop technique and phase-contrast microscopy. *Listeria* exhibit a characteristic tumbling motility.

2.2.4 Identification of the genus *Listeria* to the species level.

The haemolytic properties of some *Listeria* species may be used in combination with a Micro-ID® Listeria rapid identification system to identify *Listeria* cultures to species level within 24 h. Cultures on TSA exhibiting tumbling motility and being gram-positive, catalase positive and oxidase negative were chosen and the following tests were performed.

i) ß-haemolysis: Cultures on TSA were subcultured onto layered 6% horse blood plates (CM55 + SR50, Oxoid, Unipath Ltd., Basingstoke, U.K.). Plates were incubated at 37°C for 18 h and observed for ß-haemolysis (clearing). Three *Listeria* species are haemolytic; *L. monocytogenes* is weakly haemolytic producing a zone of clearing in a narrow band extending 1-2 mm away from the colony whilst *L. seeligeri* is very weakly haemolytic with the zone only being apparent with the removal of the colony. *Listeria ivanovii* is strongly haemolytic and exhibits a clear and wide (3-5 mm) zone of haemolysis. The remaining *Listeria* species are non-haemolytic.

ii) CAMP reaction: The CAMP (Christie, Atkins, Munch-Petersen) reaction was first described by Christie *et al.* (1944) for identification of group B *streptococci*. The test uses haemolysis enhancement by the metabolites diffused into blood agar plates by ß-haemolytic strains of *Staphylococcus aureus* (NCTC 1803) and *Rhodococcus equi* (NCTC 1621) (Supplied by the CFDRA, Chipping Campden, Gloucestershire, U.K.). These two species were streaked vertically onto separate plates of sheep blood agar (CM55 + SR50, Oxoid, Unipath Ltd., Basingstoke, U.K.). The test cultures were streaked horizontally onto the plates such that they came close to the vertical streak but did not touch. Plates were incubated at 37°C for 18 h and examined for zones of enhanced haemolysis in the vicinity of *S. aureus* or *R. equi* streaks.

L. monocytogenes and *L. seeligeri* exhibit a small (2 mm) zone of ß-haemolysis in the area of ß-toxin produced by the *S. aureus* culture. *Listeria ivanovii* exhibits a wide, semi-circular arrowhead zone adjacent to *R. equi*. The remaining species do not exhibit reactions with either organism. The CAMP test is generally recognised as the main procedure for distinguishing *L. monocytogenes* and *L. innocua*.

iii) Micro-ID® rapid identification system: The Micro ID® system was developed by Organon Teknica Corp., Durham, U.S.A for the rapid identification of members of the genus *Listeria* to species level. The test utilises reagent impregnated paper discs that react with bacterial enzymes and metabolic end products to produce readily identifiable colour changes. The strip contains 15 tests, including Voges-Proskauer (VP), nitrate reduction, phenylalanine deamination, hydrogen sulphide and indol production, ornithine and lysine decarboxylation, malonate utilisation, urea and aesculin hydrolysis, beta-galactosidase activity and fermentation of xylose, rhamnose, mannitol and sorbitol.

A suspension of each test isolate was made by mixing colonies in 3.5 ml of sterile physiological (0.85% NaCl) saline to McFarland No 1 turbidity standard. The Micro-ID® strip was inoculated with 0.2 ml of culture and incubated for up to 24 h at 37°C. Addition of KOH and analysis of the test strips were performed according to manufacturers directions. A five digit octal code was generated for each isolate and was recorded. The octal code was used in combination with ß-haemolysis and CAMP test

results to identify *Listeria* isolates to species level.

2.3 Maintenance of bacterial cultures.

Bacterial cultures described in Section 2.1 were subcultured from TSA following species confirmation (Section 2.2) and maintained on Nutrient agar slopes at 4°C. All isolates were subcultured onto fresh Nutrient agar slopes at 2 month intervals.

For long-term storage, isolates were grown overnight in 20 ml of Nutrient broth at 30°C. Following incubation, 10 ml of sterile glycerol (Analar, BDH, Poole, U.K.) was added to the culture. The culture/glycerol mixture was then vortexed for 30 s before a 2 ml aliquot was removed and placed in cryotubes and stored at -80°C until required. To retrieve cultures, a 0.1 ml aliquot from each of two temporarily defrosted cryotubes was spread over TSA. Plates were incubated at 30°C for up to 48 h and confirmed to species level (Section 2.2).

2.4 Culture of *Listeria* cells and preparation of cell inoculum.

2.4.1 Cell culture media.

During this study, the culture of *Listeria* cells for the production of cell inoculum was performed using both broth and agar medium. Two complex broth media and one agar medium were used, namely, Tryptone Soy broth (TSB) (Oxoid, Unipath Ltd., Basingstoke, U.K.), Brain Heart Infusion broth (BHI) (Oxoid, Unipath Ltd.) and Tryptone Soy agar (TSA) (Oxoid, Unipath Ltd.). Of these three media, TSB was used predominantly throughout this study.

Listeria isolates were subcultured from Nutrient agar slopes into either a 20 ml volume of broth medium or onto agar plates using streak plate technique. The composition of each of all growth media are detailed below (see Appendix A for further details of media preparation).

Tryptone Soy broth (TSB).

Per litre of distilled water, pH 7.3, 17.0 g pancreatic digest of casein, 3.0 g papain digest of soybean meal, 5.0 g sodium chloride, 2.5 g di-basic potassium

phosphate, 2.5 g glucose.

Brain Heart Infusion broth (BHI).

Per litre of distilled water, pH 7.3, 12.5 g calf brain infusion solids, 5.0 g beef heart infusion solids, 10.0 g proteose peptone, 2.0 g glucose, 5.0 g sodium chloride, 2.5 g di-sodium hydrogen phosphate.

Tryptone Soy agar (TSA).

Per litre of distilled water, pH 7.3, 15.0 g tryptone, 5.0 g peptone, 5.0 g sodium chloride, 15.0 g agar.

2.4.2 Construction of a growth curve for *L. monocytogenes*.

Standard growth curves for *L. monocytogenes* cells grown in TSB and BHI at 10°C were constructed using optical density ($O.D._{420}$) measurements from batch cultures over a 100 h incubation period. For each broth type, five replicate 20 ml volumes were inoculated (Section 2.4.1) at 0 h and at four additional time points, each staggered by a consecutive 20 h period. All broth cultures were incubated at 10°C. At 5 h intervals during incubation a 0.5 ml sample of broth was removed and its $O.D._{420}$ measured using a Cecil CE202 spectrophotometer. Each 0.5 ml sample was then discarded. A maximum of 4 samples (totalling 2.0 ml) was therefore removed from each 20 ml culture before the next staggered time sample was used. When grown in TSB at 10°C *L. monocytogenes* cells were in early-exponential, mid-exponential and early-stationary phase of growth after approximately 24, 40 and 72 h of incubation respectively (Appendix B). Early-stationary phase cells were used for production of cell inoculum unless otherwise stated.

2.4.3 Preparation of cell inoculum.

Listeria cells incubated in broth culture to early-stationary phase (Sections 2.4.1 and 2.4.2) were harvested by centrifugation at 10°C at 1800 *g*. The supernatant was discarded and the cells were resuspended in 20 ml of sterile distilled water. Cells were

washed with vortexing and again collected by centrifugation. The washing procedure was then repeated x2. Cells were finally suspended in the required diluent (explained for each separate experiment in Chapter 3) to a known cell concentration using optical density ($O.D._{420}$) measurements and a calibration curve as explained in Section 2.5.1.

All cell suspending diluents were prepared using sterile deionized water (pH 7.1) (Milli-Q reagent grade water systems; Millipore corporation, Bedford, Mass., U.S.A).

2.4.4 Preparation of ultraviolet irradiated inoculum.

A 10 ml volume of *L. monocytogenes* cells suspended in 1/4 strength Ringer's solution to an $O.D._{420} = 0.55$ was prepared (Section 2.4.3) and transferred to a sterile Petri dish. The suspension was irradiated for six consecutive 30 s intervals using an ultraviolet lamp (Model R-52G, UVP inc., San Gabriel, U.S.A) set at 200-250 V, 50-60 Hz which was placed 8 cm above the open Petri dish. During irradiation the cell suspension was continuously stirred using a magnetic flea.

Viability of the cell suspension was tested, prior to irradiation and following each 30 s irradiation period using viable cell counts. Each viable count was performed on 10^0 to 10^{-6} dilutions of the cell suspension using a spread-plate technique (Section 2.5.2) with TSA as the growth medium. Plates (3 replicates per dilution) were incubated for 48 h at 37°C. It was determined that an irradiation period of 2 min was sufficient to reduce the cell viability to an undetectable level (This corresponds to a $\geq$ 99.99% decrease in viability from the original cell suspension).

2.5 Bacteriological counts.

2.5.1 Total cell counts.

To calculate the total number of bacterial cells ml^{-1} in suspension, a Helber counting chamber and phase-contrast microscope (Reichert Bio Var) were used. For bacterial isolates which were used regularly, standard absorbance curves were prepared using a Cecil CE202 spectrophotometer at a wavelength of 420 nm ($O.D._{420}$), plotted against cell counts which were made using the Helber chamber.

2.5.2 Viable cell counts.

Dilutions of bacterial suspensions were prepared in 9.0 ml volumes of sterile 1/4 strength Ringer's solution. To calculate the viable number of cells ml^{-1} in bacterial suspensions, two basic methods were used.

i) Spread-plate technique.

Agar plates were inoculated with 0.1 ml of bacterial suspension which was spread over the entire plate surface. The plates were allowed to dry before incubation for 24 and 48 h at 37°C. The number of colonies was counted with the aid of a Gallenkamp colony counter.

ii) Dilution-spot technique.

Plates were inoculated with a dilution series of 20 μl spots. A maximum of 14 spots per plate were inoculated and allowed to dry before being incubated at 37°C for 24 and 48 h. Plates were then examined with the aid of a plate enlarger and the number of colonies growing from the appropriate dilution spots were counted.

2.6 Transmission electron microscope (TEM) examination of negatively stained *Listeria* species.

A negative stain TEM protocol was used to examine *Listeria* species for the presence of possible cell-surface appendages such as flagella, fimbriae and pili.

2.6.1 Culture of *Listeria* species for TEM examination.

Listeria species including *L. monocytogenes* isolates CRA 433, ATCC 23074, CRA 5246 & F139, *L. innocua, L. seeligeri, L. welshimeri* and *L. ivanovii* were subcultured from Nutrient agar slopes into TSB, and onto TSA plates using spread plate technique (Section 2.4.1). Plates and broth were incubated at both 10 and 25°C.

Throughout the incubation period, agar plates were examined using the Henry Illumination system (Section 2.2.2) to determine the time point at which the typical colony morphology of *Listeria* cells (grey-blue colonies having a ground glass

appearance) became visible. At this point cells were eluted from the agar surface and suspended in 10 ml sterile distilled water. Cells were collected from broth in early-stationary phase (Section 2.4.3) and suspended in 10 ml of sterile distilled water.

One ml of each culture was transferred to an Ependorf tube and the cells pelleted by centrifugation at 10,000 *g* for 5 min. The cells were washed twice in sterile distilled water followed by centrifugation. All cells were finally suspended in a 0.5 ml volume of sterile deionized water.

2.6.2 Negative staining preparation of *Listeria* cells.

i) Grid preparation.

Copper 200 TEM grids (Agar Scientific, Essex, U.K.) were coated with 2% (w/v) formvar (Analar, BDH, Poole, U.K.) in chloroform. Grids were carbon-coated (Turbocoater E6200, Biorad, U.K.) and plasma-glow discharged hydrophilic (PT7150 Plasma Barrel Etcher, Fisons Instruments, U.K.) for 2 min with air as the intake gas. This glow-discharge treatment produced a hydrophilic surface which facilitated even spreading of the stain.

ii) Negative staining.

Drops (0.05 ml) of washed cell suspension (prepared as detailed in Section 2.6.1) and 1% (w/v) methylamine tungstate (Agar Scientific, Essex, U.K.) were placed onto the copper grids using a flame stretched Pasteur pipette. After a period of 10 s, grids were blotted from one edge using Whatman No.50 filter paper, and air dried overnight in a sealed Petri dish. Samples were viewed in a Phillips 300 or Hitachi H600 TEM at 100 KV, and cells examined for the presence of surface appendages.

A negative stain preparation of the positive control organism *Streptococcus sanguis* CN3410 (White & Niven) was also prepared after 24 h incubation in BHI broth at 37°C. This Gram-positive organism has previously been shown to produce adhesive structures using these culture conditions by Handley, Carter & Fielding, (1984). Although this organism was described by these authors as being *Streptococcus salivarius*, it was later identified as *S. sanguis* biotype II according to the work of

Facklam (1977) (P. Handley. personal communication).

2.7 Scanning electron microscope (SEM) examination of artificially inoculated cabbage and lettuce leaf tissue.

Scanning electron microscopy was used to investigate the pattern of distribution and level of total adhering *L. monocytogenes* cells on artificially inoculated cabbage and lettuce leaf surfaces. Discs of leaf tissue were prepared (Section 2.7.1) prior to inoculation with drops of *L. monocytogenes* cells (Section 2.7.2). Following a period of incubation, leaf discs were washed to remove non-adhering 'planktonic' *Listeria* cells (Section 2.7.3) and prepared for examination under a SEM (Section 2.7.4). Organisms which were not removed from the leaf tissue by the standard washing procedure were termed 'adhering'. Such adhering organisms were quantified from SEM images as detailed in Section 2.7.5.

2.7.1 Selection and storage of leaf tissue for inoculation with *L. monocytogenes.*

i) Selection of leaf tissue and preparation for inoculation.

Whole Dutch white cabbage and Iceberg lettuce were purchased from a local greengrocers. Samples of cabbage and lettuce which exhibited any symptoms of internal rot were discarded. In an attempt to avoid the presence of natural micro-flora on the salad vegetables, 3 layers of outer leaves were removed, from each cabbage and lettuce sample and discarded. Internal leaves of samples were selected on the basis of being relatively unfolded and flat. Selected leaves were detached from the stem using a sharp knife and placed adaxial surface downwards onto a sterile cutting board. Using a flame sterilised 1.5 cm diameter cork borer, individual leaf discs were cut, from inter-veinal areas near the periphery of each leaf.

ii) Storage of leaf tissue in a humidity chamber.

A humidity chamber was developed for the storage of leaf tissue prior to inoculation with bacterial cells and, following inoculation, throughout a 3 d incubation period at 10°C. The humidity chamber consisted of a transparent plastic tray which

measured 24 x 24 cm in width, 2 cm in depth and had an overlapping plastic lid. A 24 x 24 cm piece of wet paper tissue and a 20 x 20 cm piece of sterile wire gauze were placed in the base of the tray. The gauze was elevated above the tissue by 1 cm. All leaf discs were placed adaxial surface downwards onto the wire gauze. Following inoculation of the leaf discs with bacterial cells (Section 2.7.2) the humidity chamber assembly was placed between two additional empty plastic trays (this appeared to help prevent the inoculum on the leaf surface from drying, possibly by insulating the humidity chamber and maintaining a steady environment within it). All three plastic trays were then sealed within a clear polythene bag and placed into a dark 10°C cooling incubator.

The humidity chamber described above was also used in future sections for the storage of inoculated model surfaces.

2.7.2 Inoculation and incubation of leaf tissue.

L. monocytogenes CRA 433 cells were grown in TSB at 10°C until early-stationary phase, collected via centrifugation and washed x3 as described in Section 2.4.3. Cells were resuspended in deionized water or 1/4 strength Ringer's solution to an optical density $O.D._{420} = 0.56$, corresponding to a total cell concentration of 8×10^8 cells ml^{-1} (with reference to a standard absorbency graph, illustrated in Appendix B).

A 25 μl drop of cell suspension was placed onto the abaxial surface of individual leaf discs. At this stage, any discs where the inoculum spread from the centre of the disc to the edge were discarded. Control leaf discs were either inoculated with 25 μl drops of 1/4 strength Ringer's solution, or left un-inoculated as a negative control to assess the numbers of naturally occurring bacterial micro-flora present on the leaf tissue. All leaf discs were incubated at 10°C for up to 3 d within the sealed humidity chamber described in Section 2.7.1.ii.

2.7.3 Washing of leaf tissue to remove planktonic *L. monocytogenes* cells.

i) Selection of discs for washing.

Following a 3 d incubation period, triplicate inoculated leaf discs which had

clearly visible drops of inoculum remaining in the centre of the disc were selected. Any discs on which the inoculum had dried were discarded. In addition, any discs exhibiting symptoms of disease or decay were also discarded. Triplicate samples of un-inoculated discs were selected on the basis of being un-changed in appearance from 0 d. These un-inoculated control discs were not subject to the standard washing procedure which is described below.

ii) Washing of discs.

A washing procedure was developed to subject inoculated discs to a standard shear force. Using flame sterilised forceps, selected discs were held vertically downwards and transferred individually, for washing, to a universal bottle containing 20 ml of sterile distilled water. Each disc was dipped twice to the 10 ml mark, being withdrawn from the water meniscus between each dip cycle. Each disc was transferred to a further four 20 ml changes of sterile distilled water and the washing procedure repeated in each. All discs were therefore dipped a total of ten times. To remove any excess water from the disc surface, each disc was tapped delicately against the top of the final washing container.

2.7.4 Sample preparation for SEM examination.

i) Vapour fixation.

Samples were vapour fixed for 1 h using 10% (v/v) glutaraldehyde (BDH, Poole, U.K.) in 0.1 M sodium cacodylate buffer (5.39 g sodium cacodylate dissolved in 250 ml double distilled water (pH 7.3). Vapour fixation was carried out by dispensing 2 ml of fixative into a planchet, contained within a Petri dish. Samples were placed around the circumference of the planchet and the Petri dish then sealed.

ii) Freeze drying.

Following fixation, samples were frozen in liquid nitrogen slush at -208°C and transferred to a pre-cooled (-196°C) freeze-drier stage (Edwards High Vacuum Ltd., Sussex, U.K.) and maintained at -80°C and 0.001 Torr overnight. The chamber was

then raised to room temperature and samples removed.

iii) Mounting and viewing samples using SEM.

Samples were attached to an aluminium SEM stub using silver Electrodag 915 (Acheson Colloids Company, Plymouth, U.K.) and sputter-coated with gold using a Emscope Sc500 sputter coater (Biorad, U.K.). Samples were viewed using a Stereoscan 360 SEM (Cambridge Instruments, U.K.) at a working distance of 10 mm and under an acceleration voltage of 25 KV.

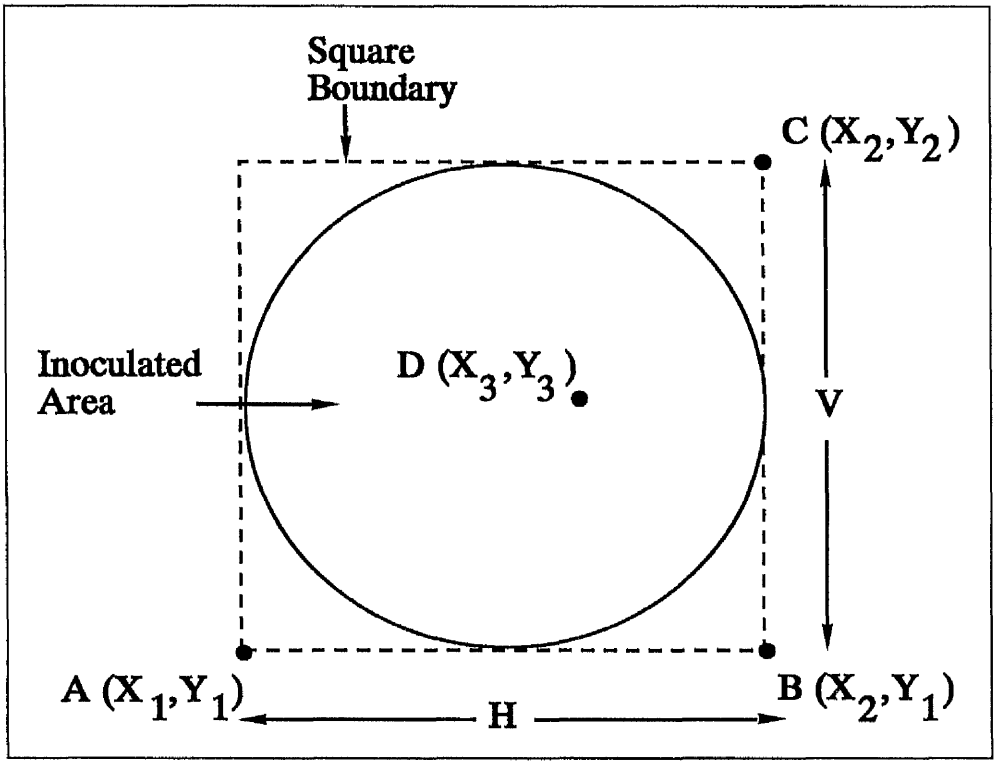
2.7.5 Quantification of total adhering bacterial flora on leaf surfaces using SEM analysis.

An SEM adhesion analysis protocol was developed to quantify the total number of adherent bacterial cells associated with the surface of inoculated leaf tissue. Per treatment, three replicate leaf discs were prepared for SEM examination as detailed in Sections 2.7.1 to 2.7.4.

i) Selection of random points within the inoculated area.

The whole inoculated area of the leaf surface was viewed at a x20 magnification. To define a square boundary surrounding the inoculated area, three reference points A, B and C, corresponding to the bottom-left, bottom-right, and top-right-hand corners of the inoculated area, respectively, were located (Fig. 2.1). These reference points were each recorded as an 8-digit XY co-ordinate using the SEM XY stage detector. The horizontal (H) displacement between points A and B, and the vertical (V) displacement between points B and C was then calculated. A random point (D) within the square boundary, in which bacteria could be quantified, was then selected as follows. The randomization function on a Casio fx-115 calculator was used to generate two independent 3-digit decimal numbers (n_1 & n_2); each number was then used to multiply either H or V to give both a random X and a Y displacement. The sample was then moved from position A by both the randomly generated X and Y displacements (Fig. 2.1). In turn, ten points ($D_{1\text{-}10}$) within the inoculated area were selected in this way.

Figure 2.1 Schematic diagram illustrating the selection of a random analysis point within a circular inoculated area of a substratum.



Reference points A, B and C mark a square boundary surrounding the area of inoculation. Each point has an 8-digit XY co-ordinate.

H = Horizontal displacement between points A and B.

V = Vertical displacement between points B and C.

$H = X_1 - X_2$ (where $X_1 > X_2$)

$V = Y_1 - Y_2$ (where $Y_1 > Y_2$)

D = The fourth randomized point selected within the square boundary.

D has co-ordinates (X_3, Y_3),

calculated as: $X_3 = X_1 - (n_1 \times H)$ and $Y_3 = Y_1 - (n_2 \times V)$

where n_1 and n_2 are two computer randomised 3-digit decimal numbers.

ii) Quantification of bacteria within each randomly selected area.

The centre of each random point, selected as described above, was viewed at a 2.0 K magnification. This showed an area on the leaf surface measuring 2200 μm^2. Each area was photographed and the number of bacteria within it was counted and converted into the number of bacteria per cm^2 of leaf surface using equation 2.1. This was performed on each of the ten random points per replicate leaf disc and a mean adhesion level for three replicate leaf discs was then calculated.

The minimum detection limit of this technique was calculated to be 4545 bacteria per cm^2. This was based on the observation that a minimum of one organism could be detected in a surface area of 22000 μm^2 for each of three replicate leaf discs (i.e. $1.0 \times 10^8/22000 = 4545$).

equation 2.1:

$$A = \frac{B \times (1.0 \times 10^8)}{2200}$$

Where, A = the calculated number of adhering bacteria per cm^2 of leaf surface and, B = the observed number of adhering bacteria in a 2200 μm^2 area of leaf tissue.

2.8 Microbiological assay to measure the adhesion and survival of *L. monocytogenes* on cabbage and lettuce leaf tissue.

A microbiological assay was developed to recover viable adhering and non-adhering 'planktonic' *L. monocytogenes* from artificially inoculated cabbage and lettuce leaf surfaces. Discs of leaf tissue were prepared (Section 2.7.1) prior to inoculation with a known concentration of *L. monocytogenes* cells (details of cell growth medium, cell diluent and cell concentration are given separately for each experiment in Chapter 3). Following incubation of leaf tissue (Section 2.7.2), leaf discs were washed (Section 2.7.3) and the planktonic *Listeria* cells recovered from leaf washings using a viable count technique (Section 2.8.1). Adhering *Listeria* cells (those not removed by the standard leaf washing procedure) were recovered using viable counts of *Listeria* by macerating the washed leaf tissue (Section 2.8.2).

2.8.1 Recovery of viable planktonic *L. monocytogenes* cells from artificially inoculated leaf tissue.

To recover viable planktonic *L. monocytogenes* cells, individual leaf discs were washed in five 20 ml changes of distilled water (total = 100 ml) (Section 2.7.3). The five washings were combined and serially diluted to a 10^{-2} dilution in sterile 1/4 strength Ringer's solution. Seven, 20 μl drops of the 10^{0}, 10^{-1} and 10^{-2} serial dilutions were plated onto LSA using a dilution spot technique. Plates were incubated at 37°C for 24 and 48 h at which points typical *Listeria* colonies on LSA were identified (as described in Section 2.2.1.i) and counted.

The viable planktonic cell population (P) recovered per disc was calculated using equation 2.2 below.

equation 2.2:

$$P = \frac{C \times D \times \text{serial diln.factor} \times 100 \text{ ml}}{7}$$

where, C = cfu per seven 20 μl drops on each agar plate, D = 50 because 50 x 20 μl = 1 ml.

2.8.2 Recovery of viable adhering *L. monocytogenes* cells from artificially inoculated leaf tissue.

To recover adhering *L. monocytogenes* cells, each washed leaf disc was transferred aseptically to a pestle and mortar and macerated using 10 ml of sterile 1/4 strength Ringer's solution. Macerates were serially diluted to a 10^{-2} dilution in sterile 1/4 strength Ringer's solution. Seven 20 μl drops of the 10^{0}, 10^{-1} and 10^{-2} dilutions were plated onto LSA using a dilution spot technique. Plates were incubated at 37°C for 24 and 48 h at which points typical *Listeria* colonies on LSA were identified (as described in Section 2.2.1.i) and counted.

The minimum detection limit of this technique was calculated to be 71.4 cfu per leaf disc. This was based on the observation that a minimum of one organism could be detected in a 140 μl volume of the 10^{0} macerated leaf sample.

The viable adhering cell population (A) recovered per disc was calculated using equation 2.3 below.

equation 2.3: $$A = \frac{C \times D \times \text{serial diln.factor} \times 10 \text{ ml}}{7}$$

where, C = cfu per seven 20 μl drops on each agar plate, D = 50 because 50 x 20 μl = 1 ml.

2.9 Cell surface hydrophobicity of *Listeria* species.

Three techniques, each described separately in this section, were used to study the hydrophobic characteristics of *Listeria* cells.

Firstly, the percentage of bacterial cells within a population which expresses a net hydrophobic nature may be calculated using the bacterial adhesion to hydrocarbon (BATH) partition assay first proposed by Rosenberg *et al.*, (1980). A modification of this protocol (described fully in Section 2.9.1) was used to determine the effect of growth medium, growth phase, growth temperature and suspending diluent on the hydrophobicity of *Listeria* cells.

Secondly, a modification of the BATH test developed by Cunniffe (1993) was used in an attempt to produce a population of predominately hydrophobic cells through a repeated enrichment procedure. The method of Cunniffe is detailed in Section 2.9.2.

Thirdly, the colloidal gold/negative stain protocol of Handley *et al.* (1991) was used to label hydrophobic and charge groups on the surface of *L. monocytogenes* cells. This protocol is outlined in Section 2.9.3.

2.9.1 Determination of cell-surface hydrophobicity of *Listeria* cells using the bacterial adhesion to hydrocarbon (BATH) test.

A modification of the original BATH test described by Rosenberg *et al.* (1980) was developed on the basis of suggestions proposed by Rosenberg (1984a). These modifications aimed to maximise the partitioning of hydrophobic from hydrophilic cell

variants and included optimising bacterial cell concentration, cell volume, hydrocarbon volume, duration of vortexing and the duration of phase separation. The optimised assay is detailed below.

Listeria species were grown in TSB and on TSA at both 10 and 25°C. Cells were collected via centrifugation and washed x3 with sterile distilled water as described in Section 2.4.3. Cells were resuspended in either deionized water, 1/4 strength Ringer's solution or 40 mM solutions of either NaCl, KCl, $NaHCO_3$, $MgCl_2.6H_2O$ or $CaCl_2$ to an optical density $O.D._{420}$ = 0.6 (= A_o).

A 4 ml aliquot of each cell suspension was dispensed into each of 10 replicate sterile glass test tubes which had been acid washed overnight in 10% (v/v) nitric acid and rinsed thoroughly with sterile distilled water. To each tube was added a 400 μl aliquot of n-hexadecane (Sigma Chemicals Ltd., St. Louis, Mo., U.S.A) and the two phase system was then vortexed for 60 s. Subsequently the tubes were left to stand for 30 min at room temperature to allow phase separation to occur.

Following phase separation, a 1 ml sample from the lower aqueous suspension of each tube was taken and its $O.D._{420}$ measured (=A). The percentage of hydrophobic cells in the original cell suspension was then calculated using equation 2.4.

equation 2.4: $$\% \text{ Hydrophobicity} = (1 - A/A_o) \times 100\%$$

Negative control test tubes were prepared by vortexing 4 ml samples of each suspending solutions (without the addition of cells) with a 400 μl aliquot of n-hexadecane. Tubes were allowed to partition for 30 min before the $O.D._{420}$ of the lower aqueous phase was measured. Any increase or decrease in optical density of the lower aqueous phase indicated inadequate phase separation due to a direct effect of the suspending diluent on the n-hexadecane (Note :this phenomenon was not encountered in any test situation used).

2.9.2 Enrichment procedure for the attempted production of a hydrophobic *L. monocytogenes* cell population. Enrichment BATH test.

The enrichment BATH test developed by Cunniffe (1993) was used in an attempt to produce a population of predominately hydrophobic *L. monocytogenes* cells. The protocol was as follows.

L. monocytogenes CRA 433 was grown in 20 ml of sterile TSB at 25°C until early-stationary phase. Cells were washed x3 in sterile distilled water and resuspended to an $O.D._{420} = 0.6$ in sterile deionized water.

A 4 ml aliquot of the cell suspension was dispensed into each of 10 sterile glass test tubes and 400 μl of n-hexadecane was added to each. The tubes were vortexed for 10 s and then allowed to partition for 30 min. This short duration of vortexing allowed only the most hydrophobic cells to partition into the upper hexadecane phase.

Following phase separation, two 20 μl samples were taken from the hexadecane layer using aseptic technique. One 20 μl sample was used to inoculate a second 20 ml quantity of TSB which was again incubated at 25°C until early-stationary phase in order to enrich the partitioned hydrophobic cells. The phase separation protocol was then repeated on these enriched cells. The second 20 μl drop was used to inoculate a TSA plate to check purity as detailed in Section 2.2. The phase separation/enrichment cycle was repeated ten times.

2.9.3 Colloidal gold labelling of hydrophobic sites on the cell surface of *L. monocytogenes*.

i) Preparation of colloidal gold.

Aliquots (0.5 ml) of 10 nM colloidal gold sol G10 (Jansen Pharmaceutical Ltd., Wantage, U.K.) having an original pH of 6.6 were dispensed into sterile bijou bottles. The pH of the colloidal gold was adjusted by the addition of either 0.1 M HCl or 0.1 M NaOH to achieve values of 3.3, 5.0, 7.0 and 10.0. A Russell Combination gelatine pH electrode (Russell pH Ltd., Fife, Scotland, U.K.) was used for this pH measurement.

ii) Labelling of *L. monocytogenes* with colloidal gold.

L. monocytogenes CRA 433 was grown in TSB at 10°C until early-stationary phase. Cells were washed x3 in sterile distilled water and resuspended in either deionized water, 1/4 or 1/2 strength Ringer's solution to an optical density $O.D._{.420}$ = 1.0. A 1 ml aliquot of each cell suspension was mixed with 0.5 ml of colloidal gold at each of the four predetermined pH values, described above. The mixture was inverted several times and the pH of the colloidal gold/bacterial mixture was again measured using a gelatine pH electrode. Mixing of the colloidal gold with the bacterial suspension resulted in changes of pH from 3.3, 5.0, 7.0 and 10.0 to 3.5, 5.2, 7.1 and 9.6 respectively. The bacteria/gold mixture was rotated on an Eschman rotary turntable at 20 rpm for 15 min at room temperature. A 1 ml aliquot of each mixture was then transferred to an Eppendorf tube and the cells pelleted by centrifugation at 10,000 *g* for 5 min. The supernatant was discarded and the cells washed in three changes of distilled water to remove un-bound colloidal gold from the cell surface. Cells were finally suspended in 0.5 ml of distilled water to a high turbidity standard. For each test condition, four negatively stained TEM samples were prepared (as described in Section 2.6.2) and each viewed for the assessment of colloidal gold labelling using a Hitachi H600 TEM.

iii) Assessment of colloidal gold labelling.

Per treatment, four replicate negatively stained TEM grids were examined using a Hitachi 600 TEM. For each grid, 50 (deionized water) and 25 (1/4 strength Ringer's solution) *L. monocytogenes* cells were examined at a 20 K magnification. The number of colloidal gold particles associated with the surface of each cell was counted by eye with the aid of a binocular viewer. A background count for non-specific labelling was estimated on each grid by counting the number of gold particles present in 10 grid areas free of *L. monocytogenes* cells.

2.10 Biophysical characterisation of leaf and model surfaces.

Two biophysical substratum surface properties namely; surface roughness and

surface hydrophobicity of five different model surfaces and, with the exception of surface roughness, two leaf surfaces, were assessed. All surfaces were prepared for analysis as detailed in Section 2.10.1 and analyzed for surface roughness and surface hydrophobicity as detailed in Sections 2.10.2 and 2.10.3 respectively.

2.10.1 Preparation of leaf and model surfaces.

i) Cabbage and lettuce leaf surfaces.

Internal leaves of Dutch white cabbage and Iceberg lettuce tissue were selected as described in Section 2.7.1. Areas of leaf tissue which were flat and free of heavy venation were chosen and a 22 x 40 mm coverslip template was laid over them. A scalpel blade was used to cut the leaf tissue around the template. Three replicate leaf strips were prepared in this way.

ii) Glass coverslip surfaces.

Glass coverslips 22 x 40 mm (Chance Propper Ltd., Warley, U.K.) were prepared using either of three protocols detailed below.

a) Coverslips were oxidised in a blue bunsen flame for approximately 2 s (FO).

b) Coverslips were dipped into 20 ml of chloroform, drained and the chloroform evaporated from the glass surface (CW).

c) Coverslips were not treated (NT) on receipt from the supplier.

iii) Cabbage-wax coated coverslip surfaces (CBW).

Jeffree, Baker & Holloway, (1975) illustrated that chloroform could be used to isolate and recrystallize the epicuticle waxes from a range of plant material. A modification of their protocol was used in this dissertation to dissolve the epicuticle wax of cabbage leaf tissue into chloroform suspension as follows.

Glass coverslip surfaces and a glass beaker were washed with chloroform as described above in part (ii.b). Cabbage leaf tissue was selected (Section 2.7.1) and a 35 g portion was placed into the glass beaker and 100 ml of chloroform was added. The beaker was then sealed and placed onto an orbital mixer for 5 min. Following mixing,

the leaf tissue was removed and disposed of. The solution was then filtered to remove debris and the chloroform removed *in vacuo*. using a rotary evaporator at 40°C. The wax remaining in the evaporator vessel was then collected, weighed and re-dissolved in chloroform to make a 0.5 % solution.

Each coverslip was then dipped ten times into a 10 ml volume of the chloroform/cabbage wax solution, the chloroform was evaporated from the glass surface between each dipping. Coverslips were examined by eye and any showing areas un-coated with a visible layer of wax were discarded.

iv) Spurr resin surfaces.

Glass coverslips were prepared as detailed in part (ii.c) and then dipped into a glass beaker containing 50 ml of hydrophobic Spurr resin (Taab Laboratory Equipment Ltd., Reading, U.K.). Each coverslip was then drained of excess resin and then supported from one of its short edges at an angle approximately 20° to the horizontal. The Spurr resin was then polymerised overnight in a 50°C oven.

v) Acetate surfaces.

Samples 22 x 40 mm of acetate photocopier transparency (Lloyd Patton Ltd., Manchester, U.K.) were cut using a glass coverslip as a template.

vi.a) Dental wax surfaces.

Strips of Anutex dental modelling wax (Associated Dental Products Ltd., Swindon, U.K.) were melted in a glass beaker by heating to approximately 60°C. Pieces of photocopier acetate approximately 40 x 70 mm in dimension were cut and then dipped once into the molten wax so that both sides of the acetate became coated with wax. The acetate was removed from the molten wax, drained, and then held in a vertical orientation until the wax coating was dry. A 22 x 40 mm glass coverslip was then used as a template to cut the wax coated acetate to the appropriate size. Surfaces were examined by eye, and any showing areas which were not coated with a visible, uniform layer of wax were discarded.

vi.b) Plasma-glow discharged dental wax surfaces (PGD).

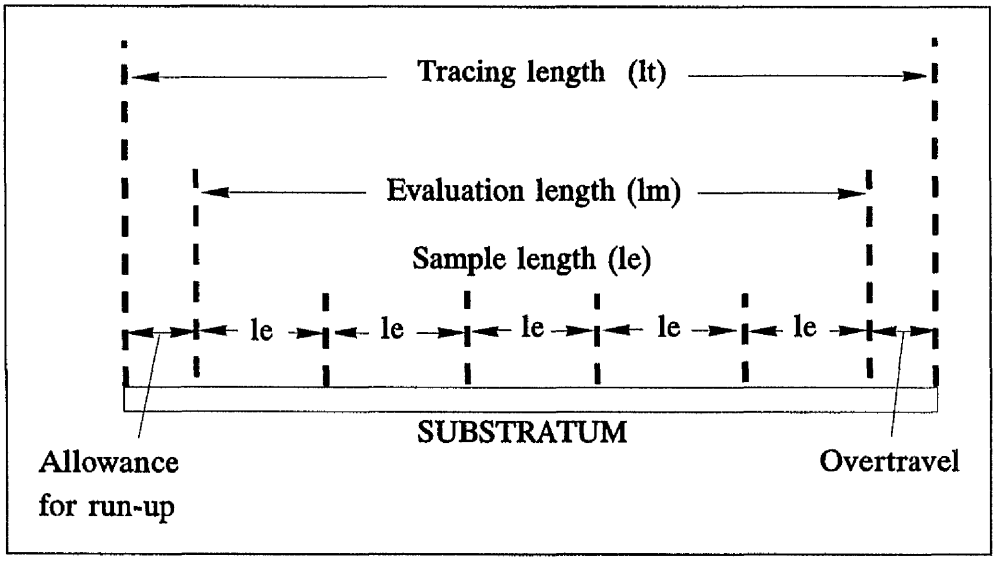
Dental wax surfaces were prepared on acetate sheet as detailed in Section vi.a above. Surfaces were then placed into the chamber of a RF Plasma Barrel Etcher (Fisons Instruments, U.K.) and plasma-glow discharged hydrophilic for 2 min using air as the intake gas.

2.10.2 Surface roughness analysis of model surfaces.

Model glass coverslip, cabbage-wax coated glass coverslip (CBW), Spurr resin, acetate, dental wax and plasma-glow discharged dental wax (PGD) surfaces were prepared as detailed in Section 2.10.1. The surfaces were laid flat onto the sample stage of a S8P Perthometer (Feinprüf Perthen GmbH, Rodenstock, W.Germany). A 17.5 x 17.5 cm analysis area with a vertical depth profile = 200 μm was defined on each surface using either of two surface roughness probes. The probe used for glass coverslip, Spurr resin and acetate surfaces was a Perthen diamond tipped stylus (FRW-750, Feinprüf Perthen GmbH, Rodenstock, W.Germany) with a stylus tip radius of 5 μm $\pm$ 2 μm and included angle of 60°. This probe was lowered to make contact with each surface with a stylus force of 0.7 mN. For softer dental wax, PGD and CBW surfaces, which because of possible surface scratching, could not be analyzed using a stylus type probe, were analyzed using a Focodyne optical probe (Feinprüf Perthen GmbH, Rodenstock, W.Germany) with a focal point diameter of 2 μm. This probe did not make contact with the substratum surface. Both probes had a vertical depth resolution of $<$ 10 nm. Surface roughness profiles for cabbage and lettuce leaf tissue could not be determined due to the large scale $>$ 200 μm variation in the depth profile over the leaf surface caused by leaf veins.

Within the 17.5 x 17.5 cm analysis area of each model surface, 129 parallel directly-traced surface roughness profiles (D-profiles) were made using either analysis probe. The probes moved over each substratum surface at a speed of 100 μm s^{-1}. Fig. 2.2 illustrates the evaluation limits for the assessment of surface roughness parameters

Figure 2.2 Schematic diagram illustrating the evaluation limits for the assessment of surface roughness parameters over a traced profile.



Tracing length (lt) = Allowance for run up + (lm) + overtravel

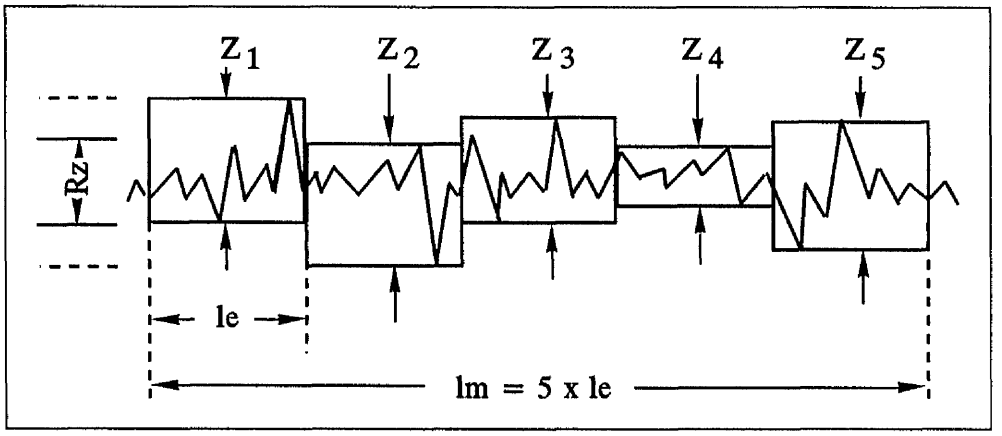
Evaluation length (lm) = 5 x Sample length (le)

(Adapted from Freeman & Richardson, 1990)

within a typical traced profile. Each profile had a 'tracing length' (lt) (actual length travelled by the probe over the surface) of 17.5 mm. This tracing length included a 1.46 mm distance at each end to allow the probe to achieve both an optimum tracing speed on run up and a distance to stop. The remaining 14.6 mm of the tracing length is termed the 'evaluation length' (lm) and it was over this distance that measurements of surface roughness were calculated. In accordance with the DIN 4768 classification (Freeman & Richardson, 1990), the evaluation length of each roughness profile was made up of five consecutive 'sampling lengths' (le) such that the tracing length totalled six times the sampling length i.e lt = 6le and lm = 5 x le.

The roughness of each model surface was defined using three profile measurements. Each measurement was derived from vertical deviations of each traced profile and measured in accordance with conditions defined by the DIN 4768 standard (Sander, 1991). These profile measurements were, Rz (mean peak to valley height), Rpm (mean levelling depth) and Ra (arithmetical mean roughness) units μm, each being calculated by averaging profile depths made over the five separate sampling lengths of the 129 parallel traces. Schematic diagrams presented in Figs. 2.3, 2.4 and 2.5 illustrate the derivation, over one line trace, of Rpm, Rz and Ra respectively.

Figure 2.3 Schematic diagram illustrating the assessment of the surface roughness parameter Rz; the mean peak to valley height made over one traced profile.

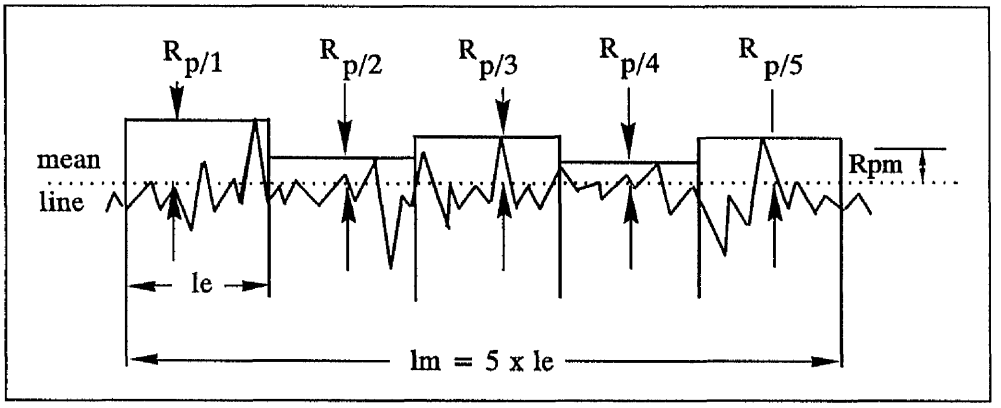


The mean peak to valley height (Rz) is the average of the single peak to valley heights of five adjoining sampling lengths (le) made throughout the evaluation length (lm).

equation 2.5. $$Rz = \frac{Z_1 + Z_2 + Z_3 + Z_4 + Z_5}{5}$$

(Adapted from Sander, 1991)

Figure 2.4 **Schematic diagram illustrating the assessment of the surface roughness parameter Rpm; the mean levelling depth made over one traced profile.**

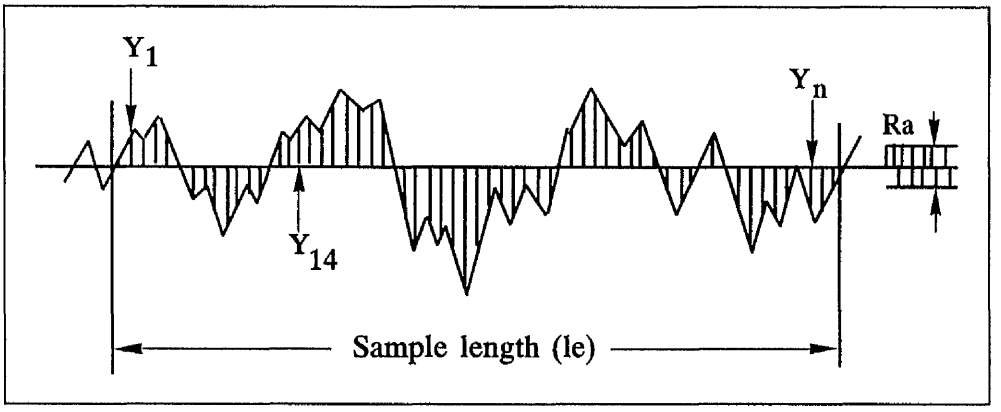


The levelling depth (R_p) is the distance between the mean line (The mean line cuts the surface roughness profile such that the profile areas above and below are equal) and a line parallel to it which passes through the highest peak of the profile within the sampling length (le). The mean levelling depth (Rpm) is the mean value of the levelling depths ($R_{p/1\text{-}5}$) of five consecutive sampling lengths throughout the evaluation length (lm).

equation 2.6. $$Rpm = \frac{R_{p/1} + R_{p/2} + R_{p/3} + R_{p/4} + R_{p/5}}{5}$$

(Adapted from Sander, 1991)

Figure 2.5 Schematic diagram illustrating the assessment of the surface roughness parameter Ra; the arithmetical mean roughness made over one traced profile.



Ra is the arithmetical average value of all departures Y of the profile from the mean line throughout the sampling length (le).

equation 2.7 $$Ra = \frac{Y_1 + Y_2 + Y_3 + Y_4 + Y_5 + Y_n}{n}$$

Y = departures of the profile from the mean line. (Simplified formula).

(Adapted from Sander, 1991)

2.10.3 Measurement of substratum contact angle.

Water contact angles were measured for leaf and model surfaces using Wilhelmy balance tensiometry (Johnson & Dettre, 1969; Neumann & Good, 1979). This technique is the basis of the Dynamic Contact angle analysis system (DCA) (Cahn Instruments, Ceriotis, California, U.S.A.). The system dynamically measures the wetting force at a solid/liquid interface as a function of immersion depth of a solid into a liquid phase. Contact angles at the solid/liquid/vapour interface are calculated automatically. A diagrammatic representation of the apparatus used in the DCA analysis system is illustrated in Fig. 2.6.i.

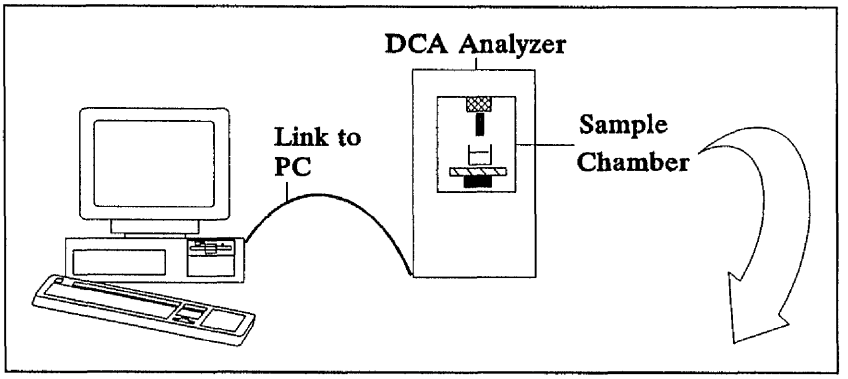
Rectangular samples of each test surface were prepared (Section 2.10.1) and attached to the electrobalance of the DCA analyzer. A 40 ml volume of double distilled water (surface tension = 72.60 dynes cm^{-1}) was placed within a clean glass beaker and positioned onto the DCA stage so that the water surface was approximately 3 mm directly below and parallel to the lowest edge of the sample (Fig. 2.6.ii.a). After taring, the beaker was automatically raised, by the computer driven stage, at a speed of 50 μm s^{-1} so that the sample became immersed in the water to a depth of 12 mm (Fig. 2.6.ii.b). At this point a dwell period of 10 s was taken to allow force readings to stabilise. The stage was then lowered (Fig. 2.6.ii.c), again at a speed of 50 μm s^{-1}, to its original position (Fig. 2.6.ii.a) to complete one immersion cycle.

The meniscus formed at the solid/liquid interface was characterised by (Θ), the dynamic contact angle. Contact angles were measured in two directions. Firstly, as the stage moves up, advancing the liquid across the solid surface (Θa). Secondly as the stage moves down, with the liquid receding across the previously wetted surface (Θr). The difference between these two contact angles is termed the contact angle hysteresis (H). Three immersion cycles were performed on each of 3 replicate samples per surface type.

During the advancing, receding and dwell stages of the immersion cycles, a buoyancy force slope was measured and recorded by the DCA analyzer. A typical one cycle buoyancy force curve is illustrated in Fig. 2.7. The point in the first immersion cycle at which the substratum first touches the water surface is termed the 'Zero Depth

Figure 2.6 Schematic diagram of the Dynamic Contact Angle analysis system.

i) Apparatus.



ii) Three actions in the measurement of a one-cycle buoyancy force curve.

a) Original sample position. b) Advancing immersion cycle. c) Receding cycle to return the sample to its original position.

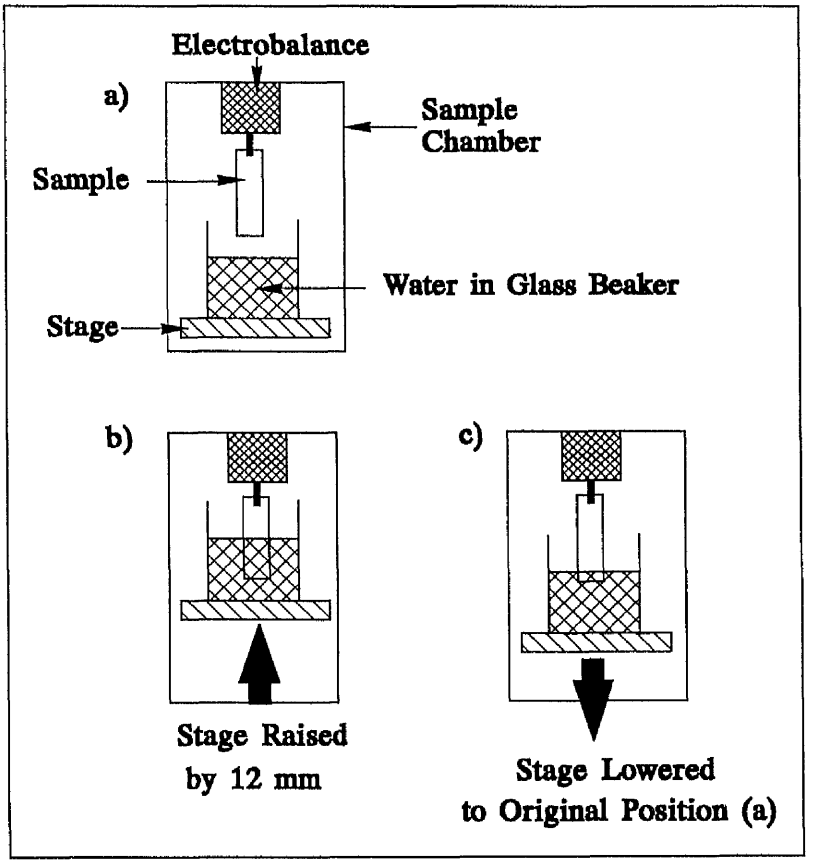
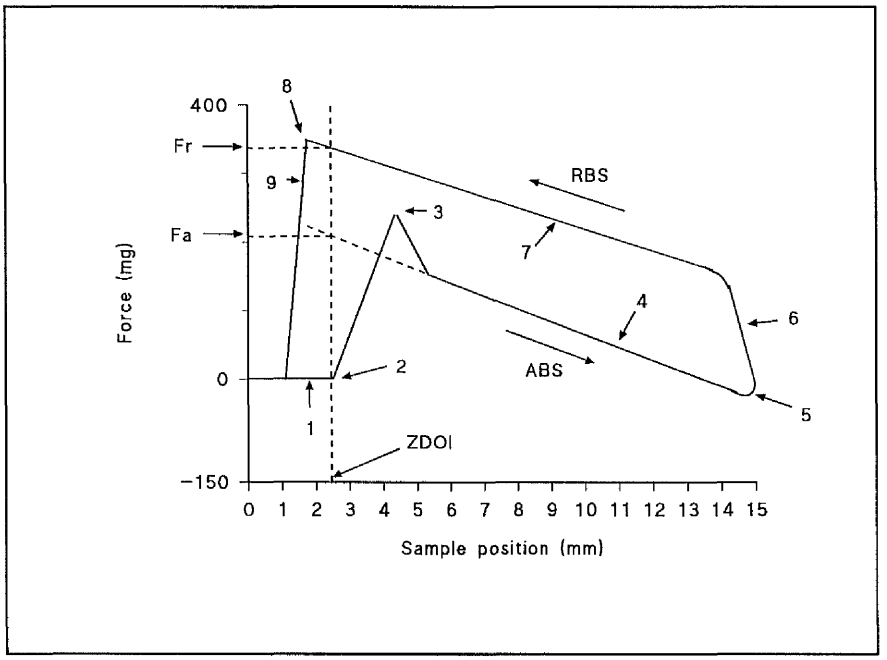


Figure 2.7 **Example of a one-cycle Wilhelmy balance hysteresis force loop showing advancing (Fa) and receding (Fr) force measurements for the calculation of advancing (Θa) and receding (Θr) water contact angles.**



The illustrated hysteresis force loop may be broken down into ten stages (0-9), namely; 0 = zero sample force measured following taring, 1 = DCA stage moves upwards, 2 = Zero Depth of Immersion (ZDOI); the water meniscus first touches the leading edge of the sample, 3 = measured sample force increases until the leading edge of sample breaks the water meniscus, 4 = water advances across and sample which experiences a force of buoyancy characterised by the advancing buoyancy force slope (ABS), 5 = dwell, 6 = stage reverses, 7 = water recedes across previously wetted sample which experiences a force of buoyancy characterised by the receding buoyancy force slope (RBS), 8 = sample exits water, 9 = stage returns to original position to complete one cycle.

Fa = force at ZDOI used to calculate advancing water contact angle Θa, Fr = force at ZDOI used to calculate receding water contact angle Θr.

of Immersion' (ZDOI). At this point the surface experiences a small force of buoyancy and a force of wetting (F) which are measured collectively by the DCA balance. Though the wetting force due to the advancing contact angle is fairly constant throughout the scan distance, a decrease in total force is recorded by the balance due to the increased buoyancy of the sample as it is immersed deeper into the water. It is at the ZDOI that calculations of advancing (Θa) and receding (Θr) contact angles were calculated automatically by the DCA analyzer using equation 2.8. Buoyancy is not part of the equation because the system uses Least Squares regression analysis (95% confidence interval) to extrapolate the buoyancy curve to the ZDOI where the wetting forces (F) for both advancing and receding cycles are calculated (Fig. 2.7).

equation 2.8. $$F = \frac{T \times P \cos \Theta}{g}$$

where:

F= Wetting force at zero depth of immersion (mg).

P = Perimeter of sample at the interface (43 mm).

T = Surface tension. Double distilled water = 72.60 dynes cm^{-1}.

Cos Θ = Cosine of contact angle.

g = acceleration due to gravity (9.8 ms^{-2}).

2.11 *In vitro* adhesion assay using total bacterial counts from SEM images to measure the adhesion of *L. monocytogenes* to model surfaces.

Initially, the adhesion of *L. monocytogenes* to seven model surfaces, namely glass coverslip [either flame oxidised (FO), chloroform washed (CW) or not treated (NT)], cabbage-wax coated coverslip, Spurr resin, acetate and dental wax was compared. Surfaces were prepared as described in Sections 2.10.1 and a set area on each surface defined (Section 2.11.1). The defined area of each surface was then inoculated with *L. monocytogenes* cells and adhesion to each surface per cm^2 assessed using total bacterial counts made from SEM images following a standard incubation and washing procedure (Section 2.11.2). Following initial experiments, the SEM adhesion

assay protocol, and an adaption of it (detailed in Section 2.12), were used exclusively to study the adhesion of *L. monocytogenes* to dental wax surfaces.

2.11.1 Preparation of model surfaces for inoculation with *L. monocytogenes.*

Glass coverslip, cabbage-wax coated coverslip (CBW), Spurr resin, acetate and dental wax surfaces were prepared as described in Section 2.10.1. Two separate techniques (detailed in parts i & ii below) were then used to define a region (area = 0.785 cm^2) on each surface into which a 500 μl volume of bacterial cells could be inoculated and confined.

i) Defining an area on glass coverslip, CBW, acetate and Spurr resin surface.

A 0.785 cm^2 area on each surface was defined using a ring of dental wax as follows: A pre-heated No. 10 cork borer was dipped once into a beaker of molten dental wax. The cork borer was then transferred quickly to the model surface and held firmly in position in contact with it. The dental wax was allowed to solidified slightly before the cork borer was removed leaving a ring of molten dental wax of 1 cm internal diameter on the surface. The wax ring was then allowed to solidify fully.

ii) Defining an area on dental wax surfaces.

A 0.785 cm^2 area on dental wax surfaces was defined using a No.10 cork borer to score a 1 cm internal diameter ring onto the surface.

2.11.2 Inoculation of model surfaces and quantification of adherent cells using SEM adhesion analysis.

i) Inoculation of surfaces and preparation for SEM analysis.

L. monocytogenes cells were grown in TSB at 10°C, washed x3 in distilled water and resuspended to a known total cell concentration in the required diluent (further details of cell-suspending diluent and inoculum concentration are given separately for each experiment in Chapter 3). Drops of 500 μl of cell suspension were then placed onto the defined area of each model surface. Surfaces were incubated at 10°C in a

humidity chamber (developed for the storage of leaf tissue and described in Section 2.7.1.ii) and sampled over a 24 h incubation period.

Following incubation, each surface was washed to remove planktonic cells by dipping 10 times into 100 ml of sterile distilled water. Surfaces were drained and then air dried for 30 min in a laminar flow cabinet. Each sample was then vapour fixed (Section 2.7.4.i), mounted on an SEM stub, sputter coated with gold and viewed using a Stereoscan 360 SEM (Section 2.7.4.iii). Three replicate samples of each surface type were prepared in this way.

Un-inoculated, negative control samples of each surface type were also prepared for use in surface topography analysis. These samples were vapour fixed directly following their production, mounted on SEM stubs and coated with gold as described previously.

ii) Quantification of adherent cells using SEM adhesion analysis.

Three replicate samples per model surface type were prepared for SEM adhesion analysis as described above. For each sample the whole inoculated zone was viewed at x6 magnification from a 10 mm working distance. As described in Section 2.7.5.i, ten random points within the inoculated area were selected. The centre of each random point was then viewed at a 2.0 K magnification which showed an area of the model surface measuring 2200 μm^2. Bacteria present in this area were counted either directly from the SEM viewing screen (for situations where bacterial numbers were $<$ 100 cells) or the area was photographed (when >100 cells were present) and bacterial counts made from photographs. The number of bacteria per area was converted into the total number of bacteria per cm^2 of model surface using equation 2.1 (Section 2.7.5).

In situations where $<$ 20 bacterial cells were present at a 2.0 K magnification, the area was viewed at a lower magnification of 1.0 K (showing an area = 8800 μm^2) and bacterial counts made directly from the viewing screen. These bacterial counts from each area were then converted into total adhering bacteria per cm^2 using equation 2.9 below.

The mean number of adhering bacteria per cm^2 for each surface type was

calculated from the average of bacterial counts made in ten random areas over each of three replicate samples i.e. a total of 30 random areas per surface type.

The minimum detection limit of this technique was calculated to be 1136 bacteria per cm^2. This was based on the observation that a minimum of 1 organism could be detected in a 88000 μm^2 area on each of three replicate samples (i.e. $1.0 \times 10^8/88000 = 1136$).

equation 2.9. $$A = \frac{B \times (1.0 \times 10^8)}{8800}$$

Where, A = the calculated number of adhering bacteria per cm^2 of model surface and, B = the observed number of adhering bacteria in a 8800 μm^2 area of each replicate sample.

2.12 *In vitro* photometric adhesion assay using crystal-violet staining of adherent *L. monocytogenes* cells on dental wax surfaces.

An *in vitro* photometric adhesion assay was developed to rapidly quantify adherent *L. monocytogenes* cells on thin layers of dental wax. The assay was based on the procedure proposed by Fletcher (1976), who fixed adherent *Pseudomonas spp.*, on polystyrene Petri dishes using Bouin's fixative, and stained the adherent cells with crystal violet. Numbers of adherent bacteria were assessed by measuring the extinction of the crystal-violet stained bacterial film against clean polystyrene.

The assay developed in this dissertation used crystal-violet staining (Section 2.12.5.ii) of fixed (Section 2.12.5.i) adherent *L. monocytogenes* cells on dental wax surfaces following a standard inoculation (Section 2.12.3) and washing procedure (Section 2.12.4). Photometric measurements of stained bacterial films were made and these calibrated against a total bacterial count of adherent cells using two independent methods which are described separately in Sections 3.6.1.i and 3.6.1.ii.

2.12.1 Production of dental wax surfaces.

Strips of Anutex dental modelling wax were melted in a glass beaker by heating to approximately 60°C. Pieces of photocopier acetate approximately 40 x 70 mm in dimension were cut, taped together and sealed back to back. The two acetate pieces were then dipped once into the molten wax so that one side of each acetate piece became coated with wax. Excess wax was then drained and the wax-coated acetate sheets held in a vertical orientation until the wax was dry. Once dry, the two pieces of wax-coated acetate were separated and examined by eye. Any surfaces showing areas which were not coated with a visible uniform layer of wax were discarded. Discs of wax-coated acetate were then cut using a hole punch with a 5 mm bore size. This produced discs coated on one side with dental wax with a surface area of 0.196 cm^2.

2.12.2 Screening of dental wax surfaces for uniformity in optical density.

Acetate discs, coated on one side with dental wax (Section 2.12.1), were placed individually into the flat bottomed wells of a 96 well (12 x 8) tissue culture plate (Falcon 3072, Becton Dickenson, U.S.A). One control row was used in each plate. Plates were read photometrically at 570 nm using a MicroELISA Auto Reader (MR 7000, Dynatech, Guernsey, U.K.). The average $O.D._{570}$ of 1000 replicate disc was calculated. Discs were sorted into batches of ten replicates by selecting discs which did not differ from the mean $O.D._{570}$ by more than $\pm$ 0.003. Ten replicate discs were used per treatment for future experiments.

2.12.3 Inoculation of dental wax discs with *L. monocytogenes* cells.

L. monocytogenes cells were grown in TSB to early-stationary phase. Cells were washed x3 in distilled water (Section 2.4.3) and resuspended to a known total cell concentration in the required diluent (explained for each experiment in Chapter 3).

Dental wax discs were sorted (Sections 2.12.2) and placed wax surface upwards onto a sterile wire gauze, contained within a humidity chamber (developed for the storage of leaf tissue and described in Section 2.7.1.ii). A 50 μl drop of cell suspension was placed onto each of ten replicate dental wax discs. Ten replicate control wax discs

were each inoculated with a 50 μl drop of the cell suspending diluent without the addition of cells. All discs were incubated within the sealed humidity chamber, at 10°C for up to 24 h.

2.12.4 Washing of dental wax surfaces to remove non-adhering 'planktonic' *L. monocytogenes* cells.

Predominantly, and unless otherwise stated in Chapter 3, inoculated discs were washed, following the required incubation period, using sterile distilled water. Two experiments (described in detail in Section 3.6.10) also examined the removal of adherent cells using the range of surface-active agents (SAA) described in part ii below.

i) Washing with distilled water.

Two washing procedures using distilled water were examined, unless otherwise stated in Chapter 3, inoculated discs were washed by dipping 10 times in 100 ml of sterile distilled water. Alternatively discs were dipped 20 times. Following washing, all discs were drained and air dried for 30 min in a laminar flow cabinet.

ii) Washing with surface-active agent (SAA).

Four categories of SAA were used, namely, Non-ionic, Anionic, Cationic and Zwitterionic. Eight SAA were selected on the basis of their common usage in the food industry.

a) Non-ionic SAA were of two structural types:- either [Polyoxyethylene Ethers:- Sorbitan monolaurate (Span 20), (S6635, Sigma Chemical Co. U.K.) and 10 Cetyl ether (Brij 56), (P5759, Sigma Chemical Co. U.K.] or [Polyoxyethylenesorbitan:- monolaurate (Tween 20), (P1379, Sigma Chemical Co. U.K.) and monooleate (Tween 80), (P1754, Sigma Chemical Co. U.K.)].

b) Anionic SAA:- [Lauryl sulphate:- Sodium dodecyl sulphate (SDS),(L5750, Sigma Chemical Co. U.K.)]

c) Cationic SAA:- [Alkyltrimethylammonium bromides:- Benzethonium chloride (BC), (B1383, Sigma Chemical CO. U.K.) & Cetyldimethylammonium bromide (CB),(C0636, Sigma Chemical Co. U.K.)].

d)ZwitterionicSAA:-[(3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate) (CHAPS), (C3023, Sigma Chemical Co. U.K.)].

Surface-active agents were prepared at 5, 10, 50, 100, 1000 & 10000 ppm concentrations in sterile distilled water.

Dental wax surfaces were dipped either 8 or 18 times in either 100 ml of SAA and then twice in sterile distilled water. Following washing, all discs were drained and air dried in a laminar flow cabinet.

2.12.5 Fixation and staining of adherent *L. monocytogenes* cells on dental wax surfaces.

i) Fixation.

Adherent cells were fixed by placing a 50 μl drop of Bouin's fixative (25 ml, 40% formaldehyde; 5 ml, glacial acetic acid; 75 ml, 25% picric acid) (Christensen *et al.*, 1985) onto the inoculated area of each wax disc. Following a 30 min fixation period, each disc was washed, delicately, by dipping 3 times into a universal bottle containing 20 ml of sterile distilled water. Dental wax surfaces were again air dried.

ii) Staining.

Fixed bacterial films were stained using a 50 μl drop of 0.5% (w/v) ammonium crystal violet (G8X, Edward Gurr, London, U.K.) which was placed onto the inoculated area of each disc. Following a 30 min staining period, each disc was again washed, delicately, by dipping 3 times into a universal bottle containing 20 ml of sterile distilled water. Dental wax surfaces were again air dried.

2.12.6 Measurement of bacterial film stain intensity.

Stained bacterial films on dental-wax coated acetate discs were placed into

individual wells of a 96 well (12 x 8) tissue culture plate. Plates were read photometrically at 570 nm using a MicroELISA Auto Reader (MR 7000, Dynatech, Guernsey, U.K.). The $O.D._{570}$ of each replicate acetate, dental wax and bacterial film complex (=C1) was recorded.

Ten control wax-coated acetate discs, each inoculated with a 50 μl drop of diluent without the addition of bacterial cells, were washed, fixed, stained and air dried in an identical manner to surfaces which were inoculated with bacterial cells. The $O.D._{570}$ of 10 control discs was measured as described previously and the average control $O.D._{570}$ calculated (=C2).

The $O.D._{570}$ of the bacterial film (B) for each replicate disc was then calculated as B = C1-C2. An average bacterial film $O.D._{570}$ was then calculated for ten replicate discs.

2.13 Statistical analysis.

Statistical analysis was performed in accordance with the procedures outlined by Woolfson (1993). Mean values were calculated with degrees of error, expressed as standard deviation values (σn-1). All data were tested for normal distribution from a calculated skewness coefficient. Normally distributed data were compared using a two-tailed Student's t-test. Parametric data from more than two sample groups were compared using one-way ANOVA. An ANOVA result that indicated a significant difference between treatments was further analyzed using a multiple-range test (Scheffe's procedure) to seek an ordering of treatments.

Statistical analysis was performed on all data by mathematical functions within Figure Perfect v.6.0c and Epistat v. 2.0. The level of significance was set at $p<0.05$ for all comparisons unless stated otherwise.

Chapter Three

Results

3.1 SEM examination of Dutch White cabbage and Iceberg lettuce leaf tissue before and after inoculation with *L. monocytogenes*.

Results outlined in this section were obtained in a preliminary study using the SEM protocol detailed in Section 2.7. The aims of this study were three-fold.

Firstly, to investigate the surface morphology of cabbage and lettuce leaf tissue and assess the level of naturally occurring bacterial micro-flora present on each (Section 3.1.1).

Secondly, to develop a protocol to deposit *L. monocytogenes* cells onto the leaf surfaces, so that, following a standard leaf washing procedure, the proportion of bacterial cells adhering to the leaf tissue could be observed (Section 3.1.2). Unless otherwise stated, adherent bacterial cells were classified as 'inoculated cells which remained in association with the substratum following the standard washing procedure of ten dips in distilled water'.

Thirdly, to develop a protocol to quantify the total adherent *L. monocytogenes* cell population on the two different tissue types. This protocol would then be used to investigate the mechanisms governing adhesion.

3.1.1 Examination of internal leaves of cabbage and lettuce tissue prior to inoculation with *L. monocytogenes* cells. Assessment of natural leaf micro-flora.

The morphology of the abaxial surfaces of cabbage and lettuce leaf tissue were investigated using SEM analysis. Leaf discs from internal tissue were cut (Section 2.7.1), and prepared for SEM examination (Section 2.7.4). Leaf tissue was not inoculated or washed at any point during preparation.

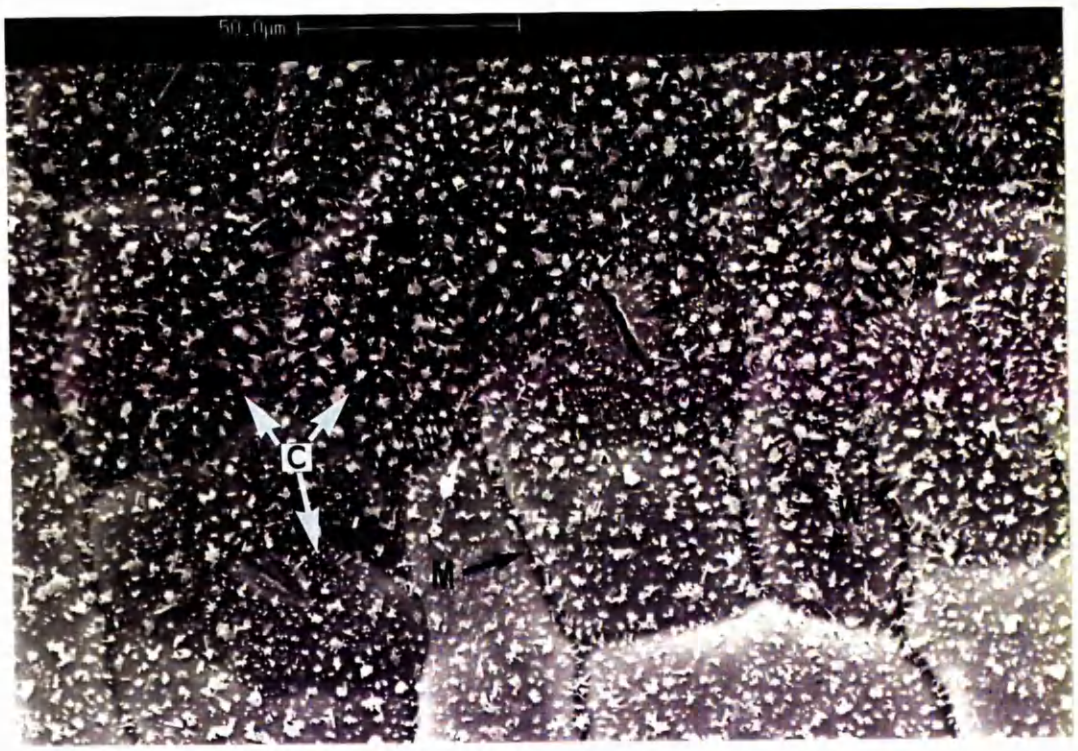
Plates 3.1 and 3.2 show examples of internal cabbage and lettuce tissue respectively (no bacterial cells were seen in either example). For both specimens, leaf cell margins and stomata are indicated. Measurement from SEM images of leaf

Plate 3.1 Scanning electron micrographs illustrating the abaxial surface of an un-inoculated internal leaf of Dutch White cabbage.

a) A 34600 μm^2 area of leaf tissue is shown. One stoma (S), epidermal leaf cells (C), leaf cell margins (M) and numerous leaf wax deposits (W) are indicated.

b) A 720 μm^2 area of leaf tissue is shown. One stoma (S) and leaf wax deposits of two structural forms are indicated. Crystalline rod-shaped wax deposits (Wc) project from the leaf surface and smooth wax deposits (Ws) spread in thin layers over the leaf surface.

a)



b)

Plate 3.2 Scanning electron micrograph illustrating the abaxial surface of an un-inoculated internal leaf of Iceberg lettuce.

A 34600 μm^2 area of leaf tissue is shown. One stoma (S), epidermal leaf cells (C) and leaf cell margins (M) are indicated. Wax deposits were not observed on this leaf tissue.

structures showed epidermal leaf cells to measure approximately 70 to 160 x 75 μm (cabbage) and 140 x 45 μm (lettuce). Stomatal guard cells measured approximately 25 x 5 μm (cabbage) and 45 x 10 μm (lettuce). In addition, cabbage tissue also possessed numerous natural leaf wax deposits. These deposits were distributed all over the abaxial cabbage leaf surface as illustrated in Plate 3.1.a. Wax deposits were of two structural types:

i) Crystalline rod-shaped wax deposits, stalagmite in appearance rising from the leaf epidermal surface. These deposits had a maximum length of approximately 4 μm (Plate 3.1.b).

ii) Smooth wax deposits rounded in shape. These deposits did not rise to a great height above the epidermal surface but appeared to spread in thin layers over it. The possibility exists that these smooth wax deposits covered the entire cabbage leaf surface in very thin layers (Plate 3.1.b).

Discrete wax deposits were not observed on any lettuce leaf samples.

The level of natural bacterial micro-flora present on internal leaves of cabbage and lettuce was assessed by direct SEM observation. Examination of 30 random areas (at a magnification of 2.0 K, as detailed in Section 2.7.5) over the surface of each of three replicate leaf discs, did not positively detect any natural leaf flora on either cabbage or lettuce tissue. Theoretically, the minimum detection level of the SEM adhesion protocol used for this assessment was calculated as 4545 cells cm^{-2} (Section 2.7.5.ii). However, due to the high numbers of wax deposits on cabbage tissue, and in some cases their similarity in shape and size to bacterial cells, it was possible that a low number of bacterial cells were miss-classified as wax deposits. In such a case, the minimum detection level would be slightly higher.

Additional control samples were also prepared by inoculating cabbage and lettuce leaf discs with 25 μl drops of either sterile deionized water or 1/4 strength Ringer's solution. These inoculated discs were incubated for 3 d at 10°C and then subject to the standard washing procedure outlined in Section 2.7.3. Discs were then prepared for

SEM examination as described previously. These discs were compared qualitatively to untreated discs to determine whether the processes of inoculation, incubation or washing had any adverse effects on the general appearance of either leaf tissue type. All inoculated discs examined were identical in appearance to un-treated discs. No illustration is shown.

3.1.2 Examination of cabbage and lettuce leaf tissue inoculated with *L. monocytogenes* cells. Quantification of total adhering cells using SEM analysis.

The experiment outlined in this section, aimed to find ways of making *L. monocytogenes* cells adhere to cabbage and lettuce leaf tissue and, subsequently, to quantify the number of adhering bacteria on each tissue type. As previously described, adhering cells were classified as inoculated cells which remained in association with the leaf surface following a standard surface washing procedure of ten dips in distilled water.

Cabbage and lettuce leaf discs were prepared from the same leaves as the control tissue described in Section 3.1.1. Inoculum of *L. monocytogenes* CRA 433 cells was prepared (Section 2.7.2) such that cells grown in TSB to early-stationary phase were finally resuspended in either deionized water or 1/4 strength Ringer's solution to an $O.D._{420}$ = 0.56. This corresponded to a total cell concentration of 8.0×10^8 cells ml^{-1} and viable cell concentration of 5.4×10^8 cfu ml^{-1} (Note: this cell concentration was used predominantly through this dissertation). A 0.08 cm^2 area of abaxial leaf tissue was inoculated with a drop of *L. monocytogenes* cells so that 2.0×10^7 cells per disc were present. For initial adhesion experiments this high inoculum concentration was used to ensure that all possible adhesion sites within the inoculated area were saturated with *Listeria* cells. Leaf tissue was incubated within a humidity chamber (Section 2.7.1.ii) for 3 days at 10°C before being washed (Section 2.7.3) and prepared for SEM examination (Section 2.7.4).

Using SEM analysis, adhesion to both tissue types did appear visually higher for cells suspended in 1/4 strength Ringer's solution compared to deionized water. Full results of SEM analysis for inoculated cabbage and lettuce leaf tissue are described

separately in Sections 3.1.2.i and 3.1.2.ii respectively. An attempt was made to quantify adhering bacterial cells on both tissue types using the SEM adhesion analysis protocol detailed in Section 2.7.5. This involved counting the number of adherent bacterial cells visible in ten random regions over the surface of each of three replicate leaf discs.

Adhesion levels to cabbage tissue are expressed in two forms in Section 3.1.2.i. Firstly, adhesion was calculated as total cells per cm^2 of leaf tissue. Secondly, for purposes of comparison with later experiments, adhesion may be calculated as cells per cm^2 and expressed as a percentage of the total cells ml^{-1} inoculated onto each leaf surface at 0 d. In this way, the number of adhering bacteria in a standard area of surface may be calculated as a percentage of the total cells in a standard volume of inoculum. Unless otherwise stated, percentage adhesion levels throughout this dissertation were calculated in this way.

For reasons described fully in Section 3.1.2.ii, quantitative aspects of adhesion to lettuce leaf tissue could not be calculated using SEM adhesion analysis. Because of this, SEM assessment of adhesion was limited to qualitative aspects. Additionally, a qualitative comparison of adhesion levels to lettuce and cabbage tissue is detailed in Section 3.1.2.iii.

3.1.2.i Adhesion of *Listeria* cells to cabbage leaf tissue.

3.1.2.i.a Localisation of adhered cells on cabbage tissue.

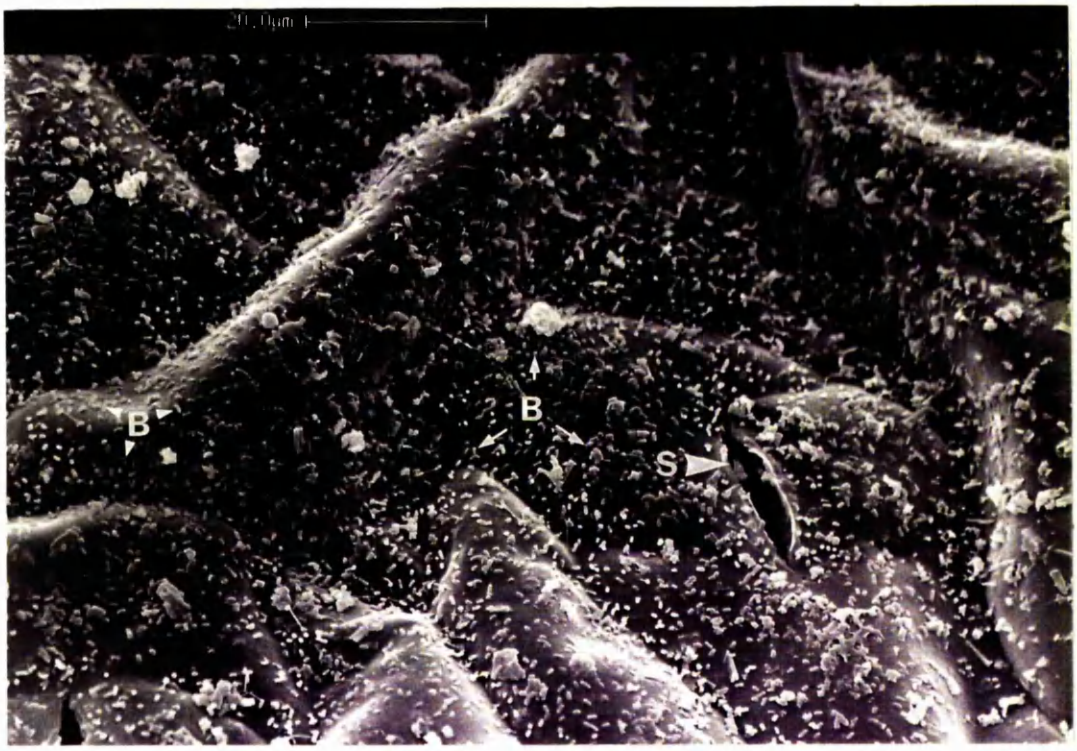
Cabbage leaf tissues inoculated with *L. monocytogenes* cells suspended in 1/4 strength Ringer's solution or deionized water and washed following a 3 d incubation period at 10°C are illustrated in Plates 3.3 and 3.4 respectively. For both cell-suspending media, adhering cells were distributed evenly over the whole inoculated area. No bacterial cells were observed outside the area of inoculation. Adhering cells were visible as a non-confluent mono-layer. Individual cells were distributed evenly over the whole leaf cell surface and not localised to leaf cell margins (Plate 3.3.a). Some bacterial cells appeared to be adhering to and forming micro-colonies around leaf wax deposits. Bacteria were, however, also observed adhering to areas of leaf tissue free from leaf wax deposits (Plate 3.3.b). The bacterial population thus appeared to

Plate 3.3 Scanning electron micrographs illustrating the abaxial leaf surface of Dutch White cabbage inoculated with *L. monocytogenes* CRA 433 cells suspended in 1/4 strength Ringer's solution. Leaf surfaces were incubated at 10°C for 3 d before being washed in distilled water.

a) A 8800 μm^2 area of leaf tissue is shown. Adhering bacterial cells (B) are distributed evenly as a non-confluent mono-layer over epidermal leaf cells. One stoma (S) is indicated.

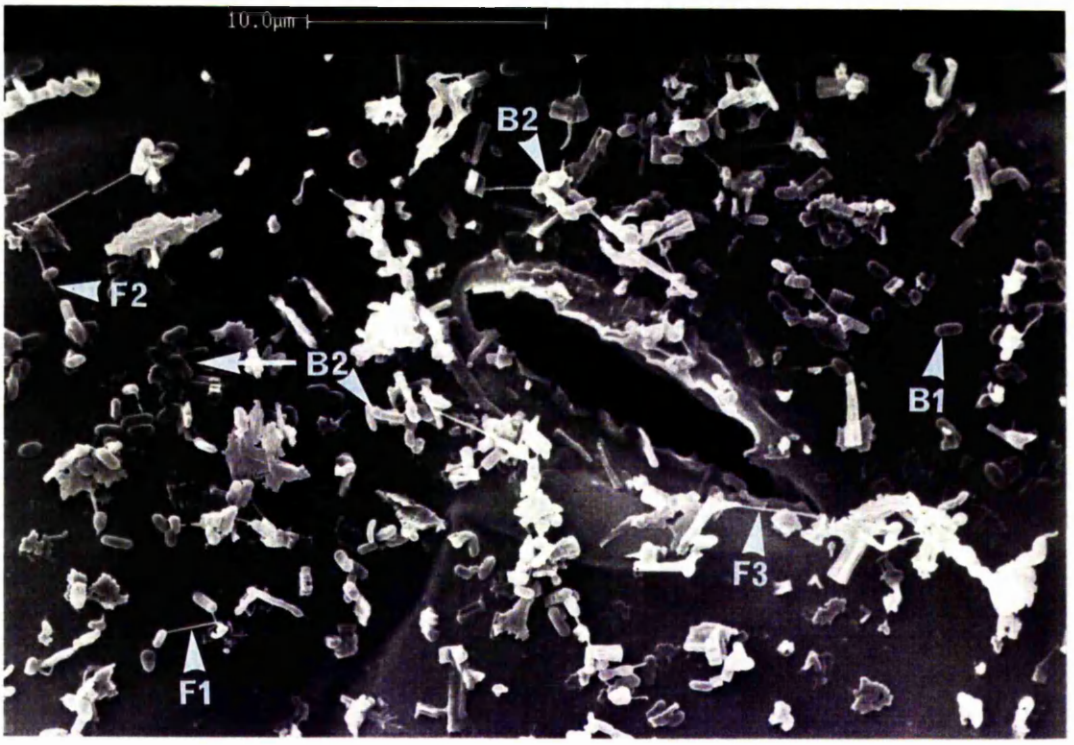
b) A 2200 μm^2 area of leaf tissue is shown in which adhering bacteria were quantified. Bacteria adhering to epidermal tissue free of discrete wax deposits (B1) and forming micro-colonies (B2) around discrete wax deposits (W) are indicated. Filaments (F) are also visible.

a)



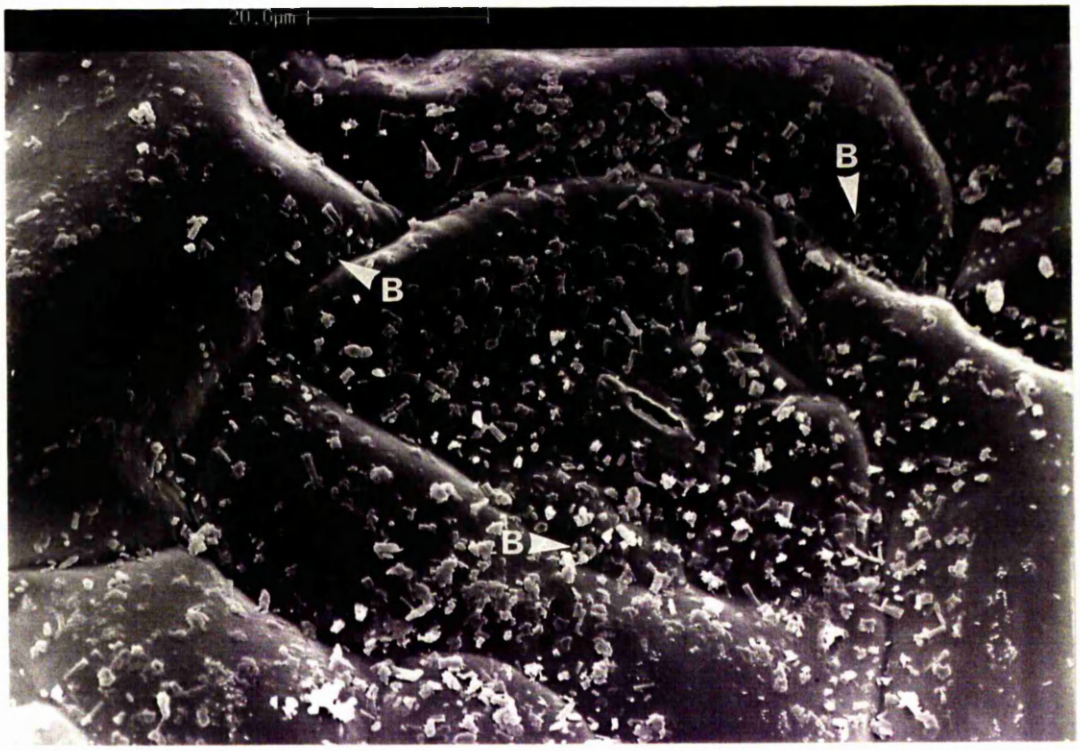
b)

Plate 3.3 continued.



c) Bacterial cells are shown in association with the epidermal leaf surface (B1) and forming micro-colonies around crystalline wax deposits (B2). Filamentous structures extending between bacteria and crystalline wax deposits (F1), bacteria and the epidermal leaf surface (F2), and individual wax deposits (F3) are also indicated.

Plate 3.4 Scanning electron micrograph illustrating the abaxial leaf surface of Dutch White cabbage inoculated with *L. monocytogenes* CRA 433 cells suspended in deionized water. Leaf surfaces were incubated at 10°C for 3 d before being washed in distilled water.



A 8800 μm^2 area of leaf tissue is shown. Low numbers of individual bacteria (B) are indicated adhering to the epidermal leaf surface.

form two discrete groupings, depending on their wax associations.

Filaments, up to 5 μm in length and approximately 0.1 μm in diameter, were often seen on cabbage leaf tissue for both cell inoculation treatments (Plate 3.3.c). These filaments were seen extending between individual bacterial cells and often connected bacterial and leaf surfaces. In addition, filaments were also seen extending between individual leaf wax deposits.

3.1.2.i.b Quantification of total adhered cells on cabbage tissue.

Quantification of adhered bacteria was made in ten random areas over the surface of each of three replicate leaf discs. Adhesion for cells suspended in 1/4 strength Ringer's solution was calculated to be 3.37×10^7 cells cm^{-2}, S.E. = 3.25×10^6 (n=3); this was significantly higher ($p < 0.05$, Student's t-test) by a factor of approximately 140 to adhesion levels for cells suspended in deionized water which was calculated as 2.41×10^5 cells cm^{-2}, S.E. = 5.3×10^4 (n=3). When expressed as a percentage of the total inoculated cells ml^{-1} (8.0×10^8 cells ml^{-1}) these adhesion levels are equal to 4.22 %, S.E. = 0.31 (1/4 strength Ringer's solution) and 0.009% S.E. = 0.001 (deionized water).

3.1.2.ii Adhesion of *Listeria* cells to lettuce leaf tissue.

3.1.2.ii.a Localisation of adhered cells on lettuce tissue.

Lettuce leaf tissues inoculated with *L. monocytogenes* cells suspended in 1/4 strength Ringer's solution or deionized water and washed following a 3 d incubation period at 10°C are illustrated in Plates 3.5 and 3.6 respectively. For both cell-suspending media, adhering bacteria were localised as a mono-layer along the margins of adjoining leaf cells, and not generally present over the main body of the leaf cells. No bacterial cells were observed outside the area of inoculation.

3.1.2.ii.b Quantification of total adhered cells on lettuce tissue.

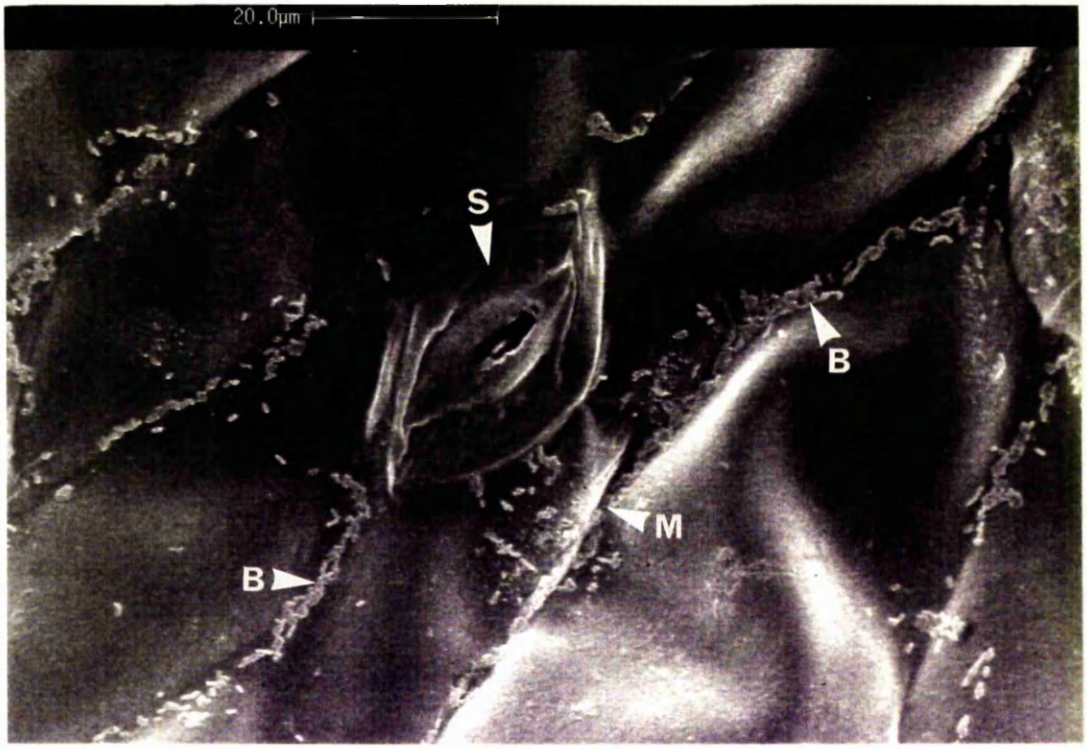
Problems were encountered when quantifying adherent cells using the SEM analysis system. These problems were the result of the localisation of *Listeria* cells

Plate 3.5 Scanning electron micrographs illustrating the abaxial leaf surface of Iceberg lettuce inoculated with *L. monocytogenes* CRA 433 cells suspended in 1/4 strength Ringer's solution. Leaf surfaces were incubated at 10°C for 3 d before being washed in distilled water.

a) A 8800 μm^2 area of leaf tissue is shown. A relatively high number of adhering bacteria (B) are distributed along the margins (M) of adjoining epidermal cells. One stoma (S) is indicated.

b) A 2200 μm^2 area of leaf tissue is shown. Bacterial cells (B) are localised along the margin of three epidermal leaf cells (E1, E2 & E3).

a)



b)

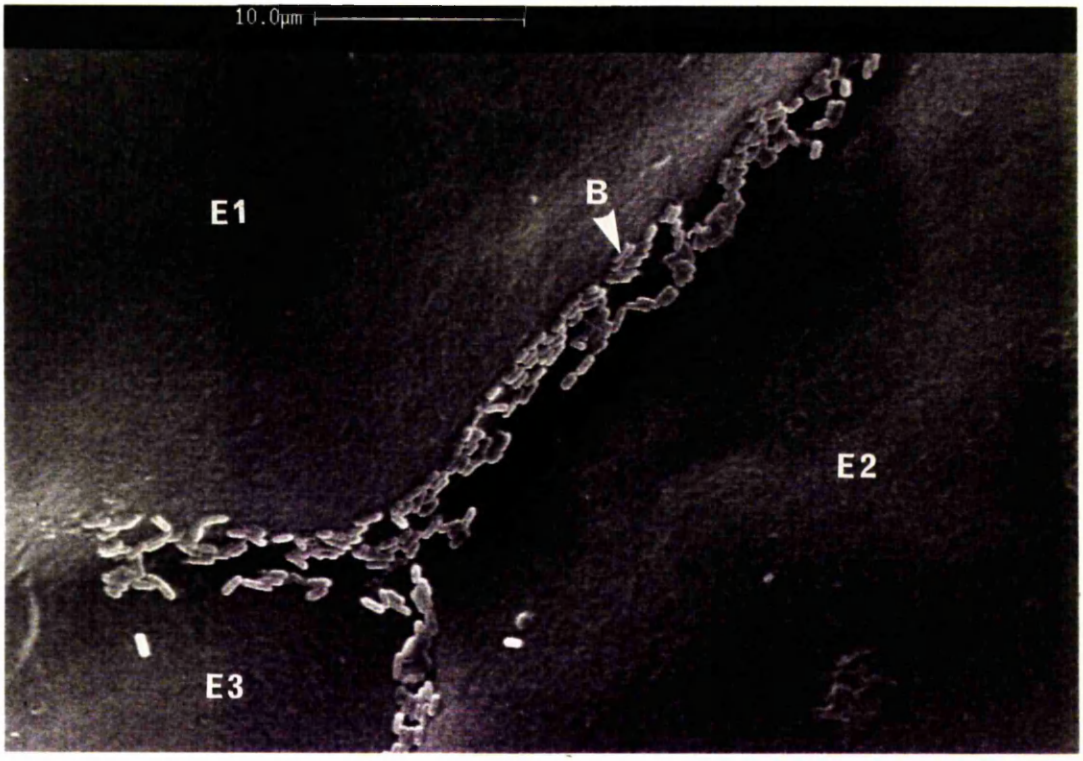
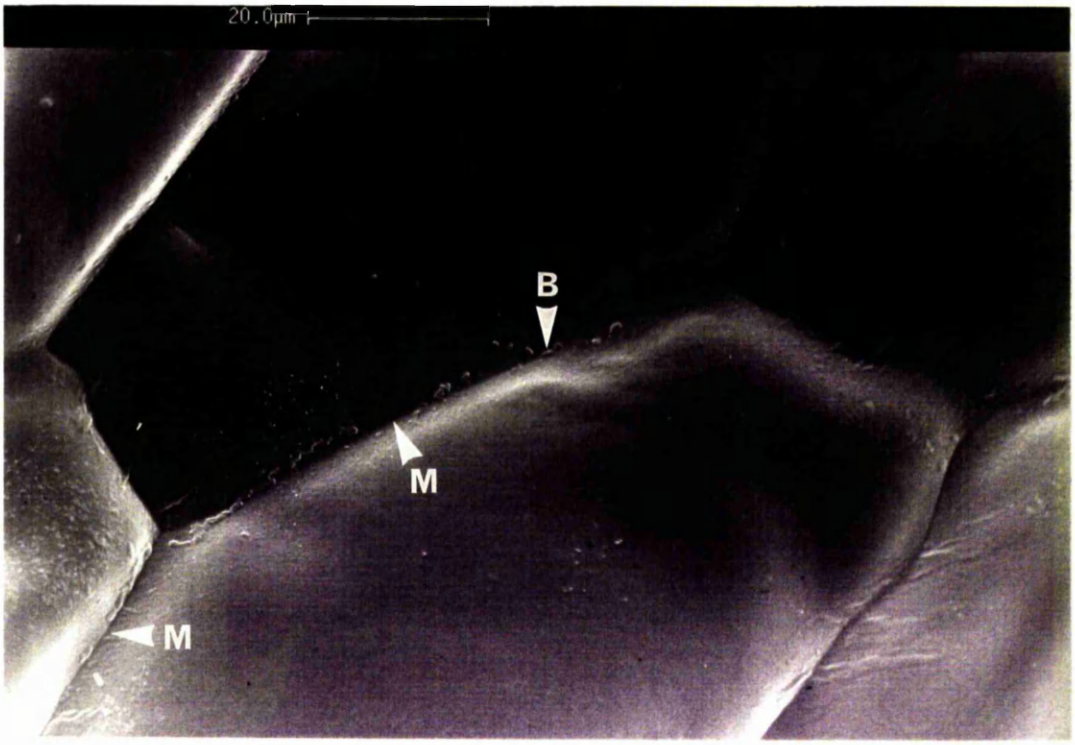


Plate 3.6 Scanning electron micrographs illustrating the abaxial leaf surface of Iceberg lettuce inoculated with *L. monocytogenes* CRA 433 cells suspended in deionized water. Leaf surfaces were incubated at 10°C for 3 d before being washed in distilled water.

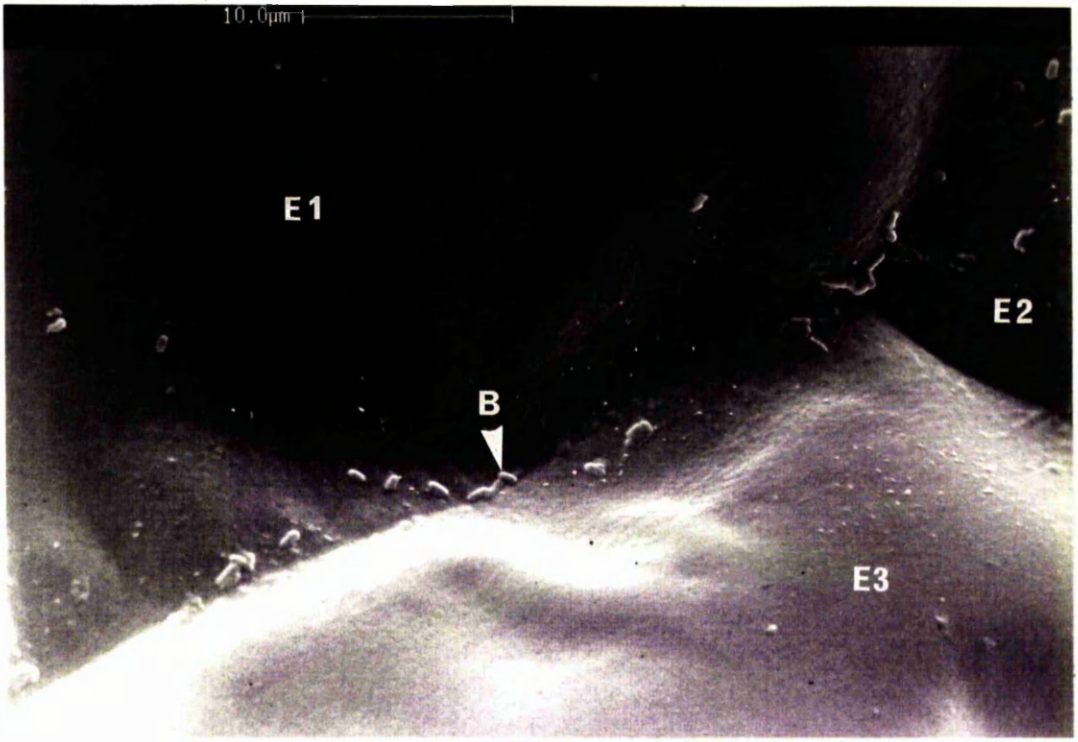
a) A 8800 μm^2 area of leaf tissue is shown. A relatively low number of adhering bacterial cells (B) are distributed along the margins (M) of adjoining epidermal cells.

b) A 2200 μm^2 area of leaf tissue is shown. Bacterial cells (B) are localised along the margin of three epidermal leaf cells (E1, E2 & E3).

a)



b)



along leaf cell margins of the tissue. Consequently, the number of adhering bacteria within an analyzed area was dependent on the number and length of the cell margins, and on the size and shape of the leaf cells in view. In order to minimise this effect, a larger area of tissue had to be analyzed at a low magnification (< 1.0 K). This was not, however, practical because at magnifications below 1.0 K individual bacterial cells became indistinguishable and impossible to count. A value for the total number of *Listeria* cells adhering per unit area of leaf surface could therefore not be reliably calculated.

When assessed qualitatively, adhesion to lettuce tissue for cells suspended in 1/4 strength Ringer's solution (Plate 3.5) did appear to be higher than adhesion for cells suspended in deionized water (Plate 3.6).

3.1.2.iii Qualitative comparison of adhesion levels on cabbage and lettuce leaf tissue using SEM micrographs.

A direct comparison of adhesion levels on cabbage and lettuce tissue, for cells suspended in 1/4 strength Ringer's solution, may be made between Plates 3.3.a and 3.5.a and, in addition, between Plates 3.3.b and 3.5.b. These sets of micrographs show identical surface areas of leaf tissue at the same magnification. The comparison indicates that, under this regime, adhesion was higher to cabbage than to lettuce tissue. For cells suspended in deionized water, a direct comparison between the two tissue types may be made between Plates 3.4 and 3.6.a. These micrographs also show identical surface areas of leaf tissue at the same magnification. This comparison indicates that when cells were suspended in deionised water adhesion levels between the two tissue types were relatively equal.

3.2 Microbiological quantification of *L. monocytogenes* adhesion to artificially inoculated cabbage and lettuce leaf tissue.

The microbiological protocol outlined in Section 2.8 was used to recover viable *L. monocytogenes* from artificially inoculated Dutch White cabbage and Iceberg lettuce leaf surfaces. The protocol aimed to monitor the viability of both adhering and non-

adhering bacterial cell populations on leaf surfaces over time. Leaf discs were inoculated with drops of *L. monocytogenes* cells and then incubated at 10°C within a humidity chamber for up to a maximum period of 3 days. Following incubation, leaf discs were washed and then macerated. Viable cells which were recovered on agar from collected leaf washing suspensions (Section 2.8.1) were termed 'planktonic'. Planktonic populations may include non-adhering cells which were in suspension above the substratum, and in addition, cells which were desorbed from the substratum by the washing procedure. Viable cells which were recovered on agar from the macerated/washed leaf tissue (Section 2.8.2) were termed 'adhering' on the basis of their resistance to being washed from the leaf surface. The 'total' viable cell recovery refers to the sum of the viable adhering and planktonic cell populations.

Four separate experiments, each using the above protocol, are detailed in this section. Three of these experiments (Sections 3.2.1, 3.2.2 and 3.2.3) monitored both planktonic and adhering cell population on lettuce leaf tissue under conditions of different cell-suspending medium, cell-growth medium and cell population level respectively. The fourth experiment (Section 3.2.4), examined the effect of cell-suspending medium on adhering and planktonic populations recovered from cabbage leaf tissue over time.

Unless otherwise stated, *L. monocytogenes* CRA 433 cells were grown to early-stationary phase in TSB and washed x3 in distilled water to produce cell inoculum (as detailed in Section 2.4). As used previously in Section 3.1, a standard cell concentration of 8.0×10^8 total cells ml^{-1} = 5.4×10^8 cfu ml^{-1} was used to inoculate a 0.08 cm^2 area of each leaf disc, so that a drop (2.0×10^7 total cells per drop = 1.35×10^7 cfu per drop) was associated with each surface. Details of cell-suspending medium are detailed fully in each separate experiment.

Data for each experiment were calculated as the average number of viable cells recovered per disc. Data are illustrated graphically to demonstrate general trends in both adhering and planktonic populations over time, and to show the relationship between the two populations. The adhering cell population may also be re-calculated as numbers of adhering cells per cm^2 of leaf surface and expressed as a percentage of the total number

of cells ml^{-1} inoculated onto the leaf surface at 0 d. In this way, and as previously described in Section 3.1.2, percentage adhesion levels may be calculated for the purpose of comparison between experiments throughout this dissertation. Such a comparison for the four experiments in this section is presented in Section 3.2.4.ii.

In order to calculate the actual proportion of the viable inoculated cells which became adhered to leaf surfaces, a 'true' percentage adhesion level may also be calculated. This was performed by dividing the viable adhered population recovered by the sum of the adhered and planktonic populations recovered per sample.

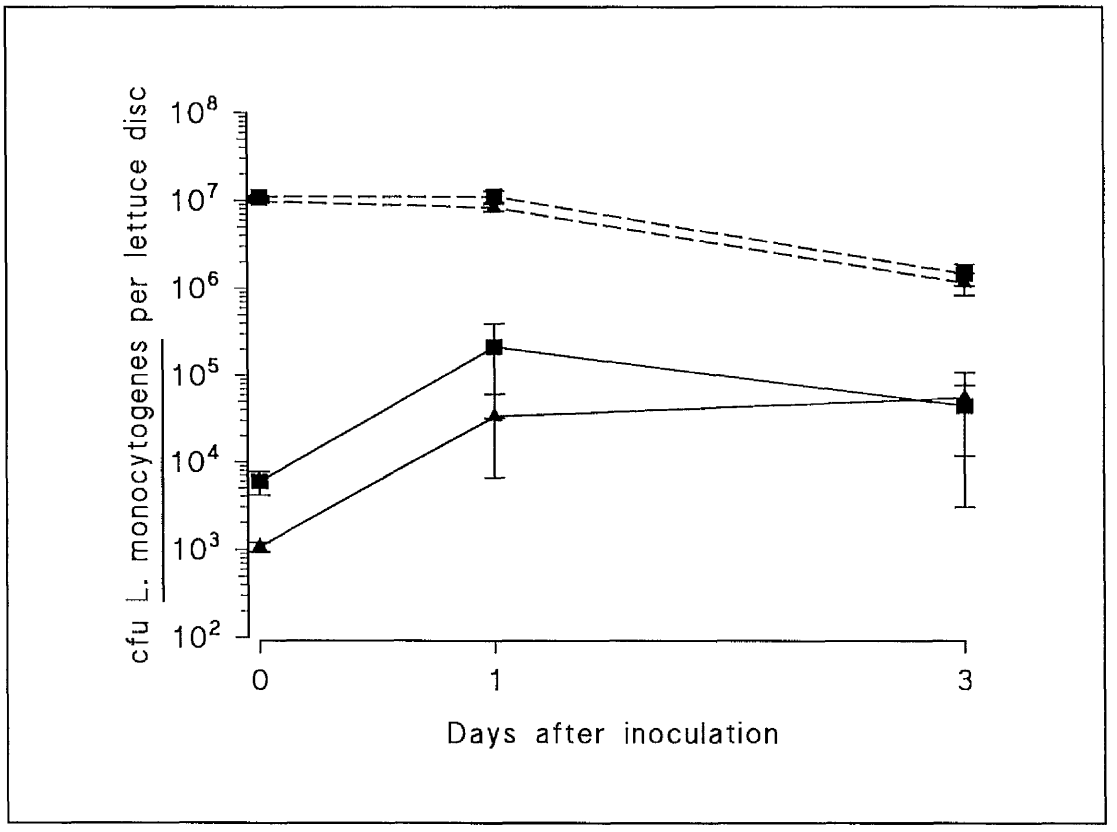
3.2.1 Effect of cell-suspending medium on the adhesion of *L. monocytogenes* to Iceberg lettuce leaf and glass coverslip surfaces.

One batch of *L. monocytogenes* CRA 433 was grown in TSB and resuspended in a 10 ml volume of deionized water. The cell batch was then divided into two equal 5 ml volumes, to one was added a further 5 ml of deionized water and to the other was added 5 ml of 1/2 strength Ringer's solution (effective electrolyte concentration therefore = 1/4 strength Ringer's solution). Two inoculum suspensions were, therefore, produced from one cell batch, each inoculum had a total cell concentration of 8.0×10^8 cells ml^{-1}.

Abaxial lettuce leaf tissue and glass coverslip surfaces [flame-oxidised (FO) as detailed in Section 2.10.1.ii.a] were inoculated (2.0×10^7 total cells per surface) with *L. monocytogenes* cells and incubated at 10°C as described previously. Lettuce surfaces were washed and macerated immediately (0), 1 and 3 d after inoculation. Glass coverslip surfaces were washed in an identical way to lettuce discs, but remaining adherent cells had to be quantified using SEM examination of vapour-fixed samples.

Figure 3.1 shows the results obtained for planktonic and adhering *L. monocytogenes* cell populations recovered from lettuce tissue at 0, 1 and 3 d. The number of adhering cells recovered increased significantly ($p < 0.1$, Multiple Range test) from 0 d, by a factor of approximately 30, to a maximum level by 1 d for both 1/4 strength Ringer's solution and deionized water. At 0 d, adhesion was significantly higher ($p < 0.05$, Student's t-test) for cells suspended in 1/4 strength Ringer's solution

Figure 3.1 Effect of cell-suspending medium on the numbers of planktonic and adhering *L. monocytogenes* cells recovered from inoculated abaxial lettuce leaf surfaces over a 3 d incubation period at 10°C.



------ Planktonic population (Number of *L. monocytogenes* cells recovered in leaf washing suspensions).

—— Adhering population (Number of *L. monocytogenes* cells recovered from the leaf macerate).

Inoculum treatments.

■ = *L. monocytogenes* cells suspended in 1/4 strength Ringer's solution.

▲ = *L. monocytogenes* cells suspended in deionized water.

Data are calculated as cells per disc for two inoculum treatments.

Values are the means of seven bacterial counts taken from either five replicate leaf discs at 0 and 3 d or six replicate leaf discs at 1 d. Bars shown are standard errors of the mean.

compared to deionized water. The difference in adhesion levels between treatments was less significant at 1 d ($p<0.1$, Student's t-test) and not significant at 3 d ($p>0.1$) due to the very large variability amongst replicate samples at this time point.

The percentage adhesion levels at 0, 1 and 3 d are expressed for both treatments in Table 3.1. When expressed in this form, the maximum average adhesion level was low and calculated as 0.33% (1/4 strength Ringer's solution at 1 d). In addition, when this maximum adhesion level was calculated as the 'true' percentage of the total viable cells recovered at 0 d, this was equal to 1.93%. This indicates that only a small proportion of the initially inoculated cells adhered to the leaf surface following incubation.

The numbers of planktonic cells recovered from lettuce tissue (Fig. 3.1) were not significantly different ($p>0.05$, Student's t-test) between the two cell-suspending treatments at either of the three sampling times. In addition, planktonic populations for both treatments remained stable over the first day of incubation and fell significantly ($p<0.05$, Student's t-test) from 1 to 3 d. This decrease was considerable; on average a drop of 9.0×10^6 cfu per lettuce disc was observed from 1 to 3 d. This decrease could not be totally accounted for by considering the population of viable cells which had adhered to the lettuce leaf surface at this time point; the maximum adhesion level observed amongst all replicate samples examined was only 1.1×10^6 cfu per disc and on average the maximum adhesion level observed was only approximately 2.2×10^5 cfu per disc.

Figure 3.2 shows the numbers of viable planktonic *L. monocytogenes* cells recovered from glass coverslip surfaces and the total viable cells recovered 'planktonic plus adhered' from lettuce leaf tissue over the 3 d incubation period. For glass surfaces, the planktonic population at each sampling time may be considered as the total viable cell population associated with the substratum. This assumption may be drawn, because SEM examination of 30 random areas (at a magnification of 1.0 K) over each of 3 replicate washed coverslip surfaces did not reveal any adhering bacterial cells, indicating that the vast majority of cells had been recovered by the washing procedure.

The four graphs shown in Fig. 3.2 illustrate identical trends in cell viability over

Table 3.1 Effect of cell-suspending medium on the adhesion of *L. monocytogenes* to lettuce leaf tissue over a 3 d incubation period at 10°C.

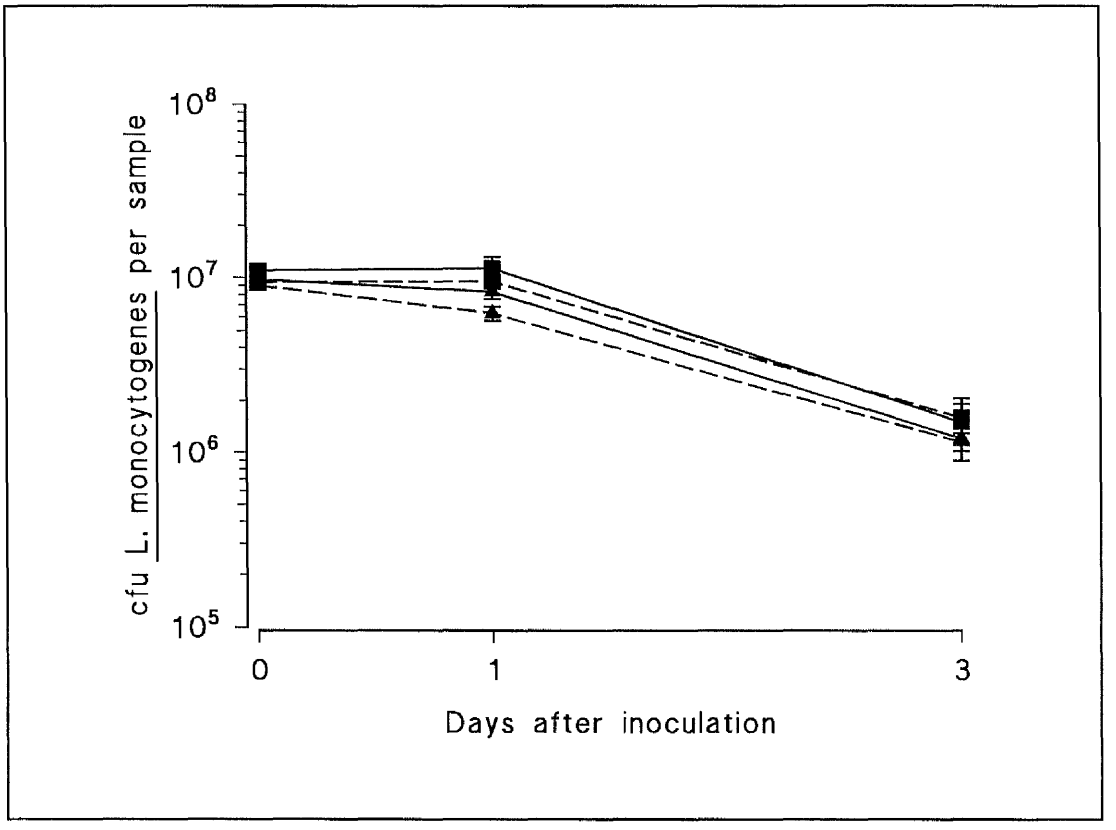
Cell Suspending Medium	% Adhesion Day 0	Day 1	Day 3
Water	0.002 ± 0.0002^{a}	0.052 ± 0.042^{c}	0.085 ± 0.083^{cd}
1/4 Ringer's	0.009 ± 0.003^{b}	0.330 ± 0.213^{d}	0.069 ± 0.050^{cd}

Data represent the numbers of viable adhering cells recovered (per cm^2) at 0, 1 and 3 d after inoculation expressed as a percentage of the total cells ml^{-1} inoculated onto the leaf tissue at 0 d.

Values are the means of seven bacterial counts taken from either five replicate lettuce discs at 0 and 3 d or six replicate lettuce discs at 1 d $\pm$ standard errors of the mean.

Values followed by the same letter are not significantly different from each other ($p > 0.1$).

Figure 3.2 Effect of cell-suspending medium on the total number of *L. monocytogenes* cells recovered from inoculated lettuce leaf and flame oxidised glass coverslip surfaces over a 3 d incubation period at 10°C.



------ Glass coverslip surfaces.

—— Lettuce leaf surfaces.

Inoculum treatments.

■ = *L. monocytogenes* cells suspended in 1/4 strength Ringer's solution.

▲ = *L. monocytogenes* cells suspended in deionized water.

Data represent cells recovered from surface washings (glass coverslips) or the mean sum of the cells recovered from leaf washing and macerate suspensions. Data is calculated as total viable cells recovered per sample for two inoculum treatments.

Values are the means of seven bacterial counts taken from either five replicate samples at 0 and 3 d or six replicate samples at 1 d. Bars shown are standard errors of the mean.

the 3 d incubation period. For each treatment, viability remained stable up to 1 d and then fell significantly ($p < 0.05$) from 1 to 3 d. At each sampling time, no significant differences ($p > 0.05$, ANOVA) existed between the four treatments.

Negative control leaf discs were also prepared to assess the numbers of naturally occurring bacterial flora present. Control discs, were inoculated with sterile 1/4 strength Ringer's solution and incubated at 10°C. These discs did not yield detectable numbers of bacterial colonies on LSA medium (<78 cfu per disc) at either 0, 1 or 3 d. This indicates that naturally occurring bacterial flora did not contribute to the data obtained from LSA medium.

3.2.2 Effect of *L. monocytogenes* growth medium on their adhesion to lettuce leaf tissue at 10°C.

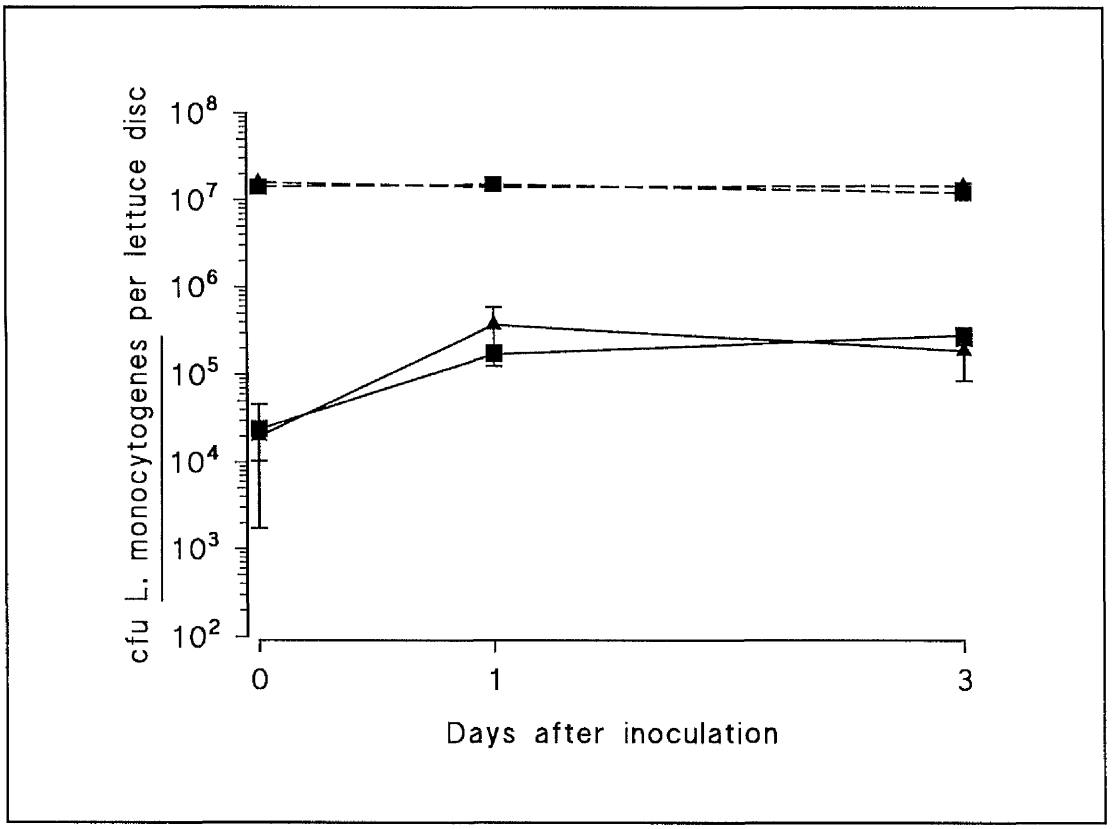
The importance of cell-growth medium on the adhesion of *L. monocytogenes* to lettuce leaf tissue was investigated. *L. monocytogenes* CRA 433 was grown at 10°C until early-stationary phase in either of two complex media (detailed in Section 2.4.1), namely, Tryptone Soy broth (TSB) and Brain Heart Infusion broth (BHI). Cells were washed x3 and resuspended in 1/4 strength Ringer's solution to a total cell concentration of 8.0×10^8 total cells ml^{-1}. The abaxial surfaces of Iceberg lettuce leaf discs were inoculated (2.0×10^7 total cells per disc) with drops of cell suspension and incubated for up to 3 d at 10°C.

Results for cells grown in TSB may be compared to results obtained in Section 3.2.1, where cells were also grown in TSB and resuspended in 1/4 strength Ringer's solution.

Figure 3.3 shows the results obtained for planktonic and adhering *L. monocytogenes* cells recovered from lettuce leaf discs 0, 1 and 3 d after inoculation. At each sampling time the planktonic populations recovered were not significantly different ($p > 0.05$, Student's t-test) between the two treatments. In addition, no significant decrease or increase ($p > 0.05$, ANOVA) was observed within either planktonic population over the 3 d incubation period.

Numbers of adhering cells recovered at 0, 1 and 3 d were not significantly

Figure 3.3 Effect of growth medium on the numbers of planktonic and adhering *L. monocytogenes* cells recovered from inoculated lettuce leaf surfaces over 3 d at 10°C. Bacteria were resuspended in 1/4 strength Ringer's solution for inoculation.



------ Planktonic population (Number of *L. monocytogenes* cells recovered from leaf washing suspensions).

—— Adhering population (Number of *L. monocytogenes* cells recovered from the leaf macerate suspensions).

Growth media.

■ = *L. monocytogenes* cells grown in Tryptone Soy broth (TSB).

▲ = *L. monocytogenes* cells grown in Brain Heart Infusion broth (BHI).

Data are calculated as cells per disc for two bacteria growth media.

Values are the means of seven bacterial counts taken from either five replicate leaf discs at 0 d or six replicate leaf discs at 1 and 3 d. Bars shown are standard errors of the mean.

different between the two growth-media treatments ($p > 0.05$, Student's t-test). Adhesion levels for both media did, however, increase significantly ($p < 0.05$, Multiple Range test) over the 3 d incubation period (Fig. 3.3). Adhesion increased to a maximum level by 1 d and did not increase significantly thereafter.

The percentage adhesion levels over the 3 d incubation period are presented in Table 3.2. For TSB at 1 d, an adhesion level of 0.268%, S.E. = 0.071, was calculated. This was not significantly different ($p > 0.1$) to the 0.33% level calculated previously on lettuce tissue at 1 d for cells grown in TSB medium and suspended in 1/4 strength Ringer's solution [Section 3.2.1 (Table 3.1)]. Percentage adhesion levels at 1 d, for both experiments, are therefore reproducible. In Section 3.2.1 however, adhesion levels at 3 d for cells suspended in 1/4 strength Ringer's solution were low and variable in comparison to levels at 1 d. This was possibly due to the large fall in viability of the planktonic population from 1 to 3 d. In this current experiment, the planktonic population did remain stable and the adhesion level of 0.444%, S.E. = 0.112 at 3 d was not significantly different ($p > 0.1$) to the 0.268% adhesion level at 1 d.

3.2.3 Effect of *L. monocytogenes* population level on their adhesion to Iceberg lettuce leaf tissue at 10°C.

Sections 3.2.1 and 3.2.2, both report measurement of the adhesion of *L. monocytogenes* CRA 433 cells to lettuce leaf tissue following inoculation with a standard total cell concentration of 8.0×10^8 cells ml^{-1} = $O.D._{420}$ of 0.56. Cells had been grown in TSB medium and resuspended in 1/4 strength Ringer's solution. Under that regime, both experiments reported the number of adhering cells to increase by a factor of approximately 30 from 0 d to a maximum number at 1 d. This maximum number was calculated as approximately a 0.3% adhesion level. The two experiments did not, however, provide any information detailing either the saturation of available adhesion sites on the substratum or possible cooperative adhesion mechanisms (either positive or negative) which may exist between bacterial cells under this cell-suspending regime. Such information may be derived using binding isotherms and Scatchard plots of adhesion data. To obtain these plots a constant amount of substratum was inoculated

Table 3.2 Effect of cell growth medium on the adhesion of *L. monocytogenes* to lettuce leaf tissue over a 3 d incubation period at 10°C.

Cell Growth Media	% Adhesion Day 0	Day 1	Day 3
TSB	0.031 ± 0.015^{a}	0.268 ± 0.071^{b}	0.444 ± 0.112^{b}
BHI	0.037 ± 0.034^{a}	0.585 ± 0.337^{b}	0.295 ± 0.164^{b}

Data represent the numbers of viable adhering cells recovered (per cm^2) at 0, 1 and 3 d after inoculation expressed as a percentage of the total cells ml^{-1} inoculated onto the leaf tissue at 0 d.

Values are the means of seven bacterial counts taken from either five replicate lettuce discs at 1 d or six replicate lettuce discs at 0 and 3 d $\pm$ standard errors of the mean.

Values followed by the same letter are not significantly different from each other ($p > 0.1$).

with several different bacterial cell concentrations and the extent of adhesion measured.

The basic premise is that T=A+P, where T is the total number of cells, A represents Adhering (Bound) cells, and P refers to the Planktonic (Unbound) population.

To obtain a range of population levels, one batch of *L. monocytogenes* CRA 433 cells was grown in TSB and washed x3, as described previously. The cells were then divided into two volumes and each resuspended in 1/4 strength Ringer's solution to produce two inoculum concentrations of optical densities $O.D._{420}$ = 1.40 (Inoculum A) and $O.D._{420}$ = 0.56 (Inoculum B) respectively. Two additional inoculum suspensions (Inoculum C and Inoculum D) were also prepared by making 10^{-1} and 10^{-2} dilutions of inoculum B.

Total and viable cell counts of inoculum suspensions A, B, C and D were made using a Helber counting chamber and spread plate technique respectively (Table 3.3). Abaxial lettuce leaf discs were then inoculated with one of the four inoculum suspensions and incubated at 10°C for up to 1 d. Planktonic and adhering populations were recovered immediately (0 d) and 1 d following inoculation.

Figure 3.4.a shows the data obtained for planktonic cells at 0 and 1 d. Each of the four inoculum concentrations produced a planktonic population which was significantly different ($p < 0.05$, Multiple Range test) to the other three at both 0 and 1 d. Increasing the inoculum concentration resulted, not surprisingly, in a proportional increase in the planktonic population recovered. Within each inoculum concentration the planktonic population remained stable throughout incubation. The total viable cell population recovered within each treatment (i.e. the sum of adhered and planktonic populations) were also not significantly different from 0 to 1 d. This indicated that no net growth or death of *L. monocytogenes* cells had occurred on the leaf surface for either inoculum concentration.

Figure 3.4.b shows the data obtained for adhering cells at 0 and 1 d. At 0 d, a detectable number of adhering cells were present for all inoculum treatments. Adhesion increased significantly from inocula D to C to B but was not significant between B and A. An identical trend was also observed at 1 d.

Within each inoculum treatment, adhesion increased significantly, by a factor of

Table 3.3 *L. monocytogenes* CRA 433 inoculum concentrations used to inoculate Iceberg lettuce leaf tissue in Section 3.2.3.

Inoculum Suspension	Total cells ml^{-1}	cfu ml^{-1}	cfu per lettuce disc
A	4.0×10^9	$3.1 \times 10^9 \pm 4.2 \times 10^8$	7.52×10^7
B	8.0×10^8	$5.4 \times 10^8 \pm 5.3 \times 10^7$	1.35×10^7
C	8.0×10^7	$5.1 \times 10^7 \pm 3.4 \times 10^6$	1.28×10^6
D	8.0×10^6	$3.6 \times 10^6 \pm 6.3 \times 10^5$	9.0×10^4

Values for viable cells are the means of six bacterial counts taken from three replicate samples within one inoculum $\pm$ standard deviations.

Figure 3.4 Effect of population level on the numbers of planktonic and adhering *L. monocytogenes* cells recovered from inoculated lettuce leaf tissue over 1 d at 10°C. Bacterial cells were suspended in 1/4 strength Ringer's solution for inoculation.

Inoculum concentrations.

■ = Inoculum A (4.0×10^9 total cells ml^{-1}).

▲ = Inoculum B (8.0×10^8 total cells ml^{-1}).

▼ = Inoculum C (8.0×10^7 total cells ml^{-1}).

♦ = Inoculum D (8.0×10^6 total cells ml^{-1}).

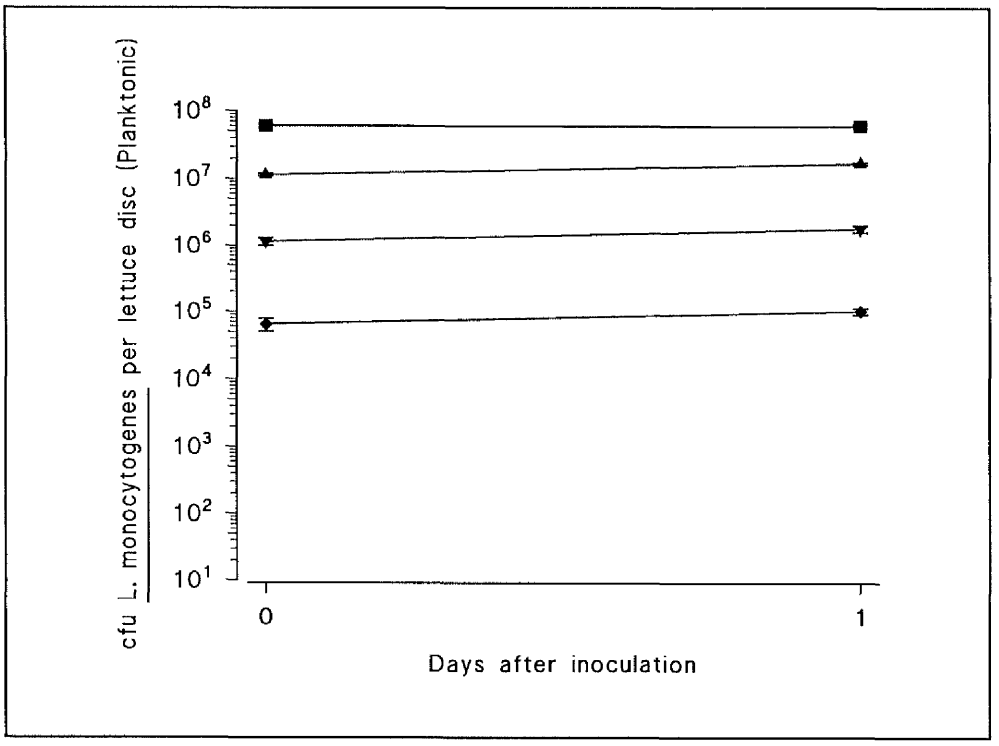
a) Planktonic population (Number of *L. monocytogenes* cells recovered in leaf washing suspensions).

b) Adhering population (Number of *L. monocytogenes* cells recovered from the leaf macerate).

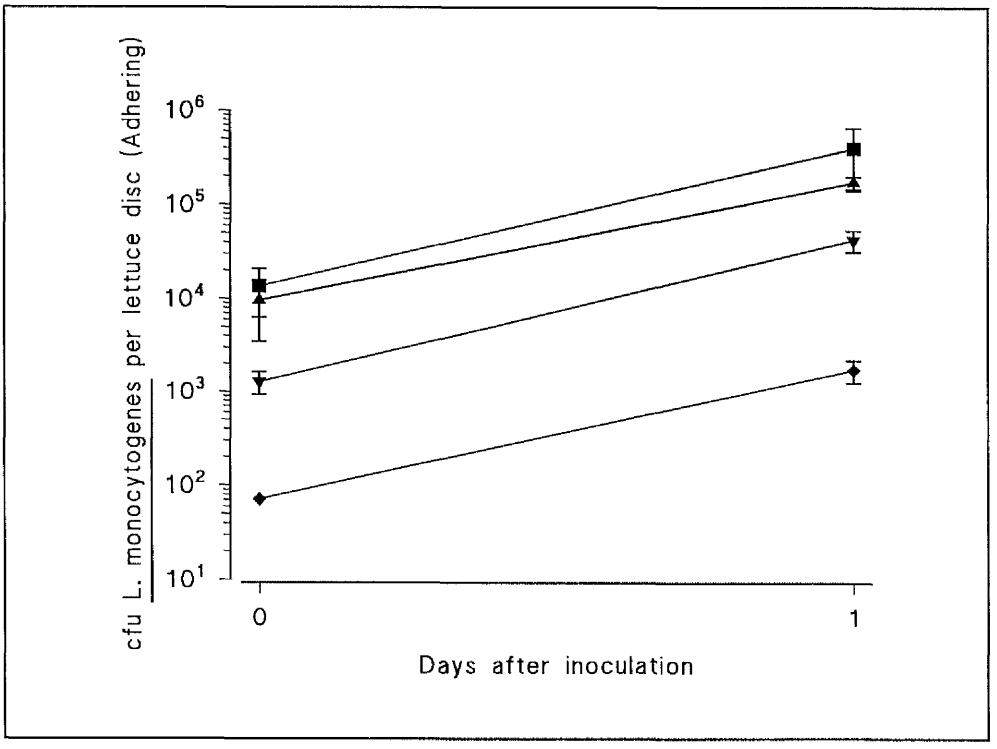
Data are calculated as cells per disc for four inoculum concentrations.

Values are the means of seven bacterial counts taken from each of seven replicate lettuce discs. Bars shown are standard errors of the mean.

a)


cfu L. monocytogenes per lettuce disc (Planktonic)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
1
Days after inoculation

b)


cfu L. monocytogenes per lettuce disc (Adhering)
10^6
10^5
10^4
10^3
10^2
10^1
0
1
Days after inoculation

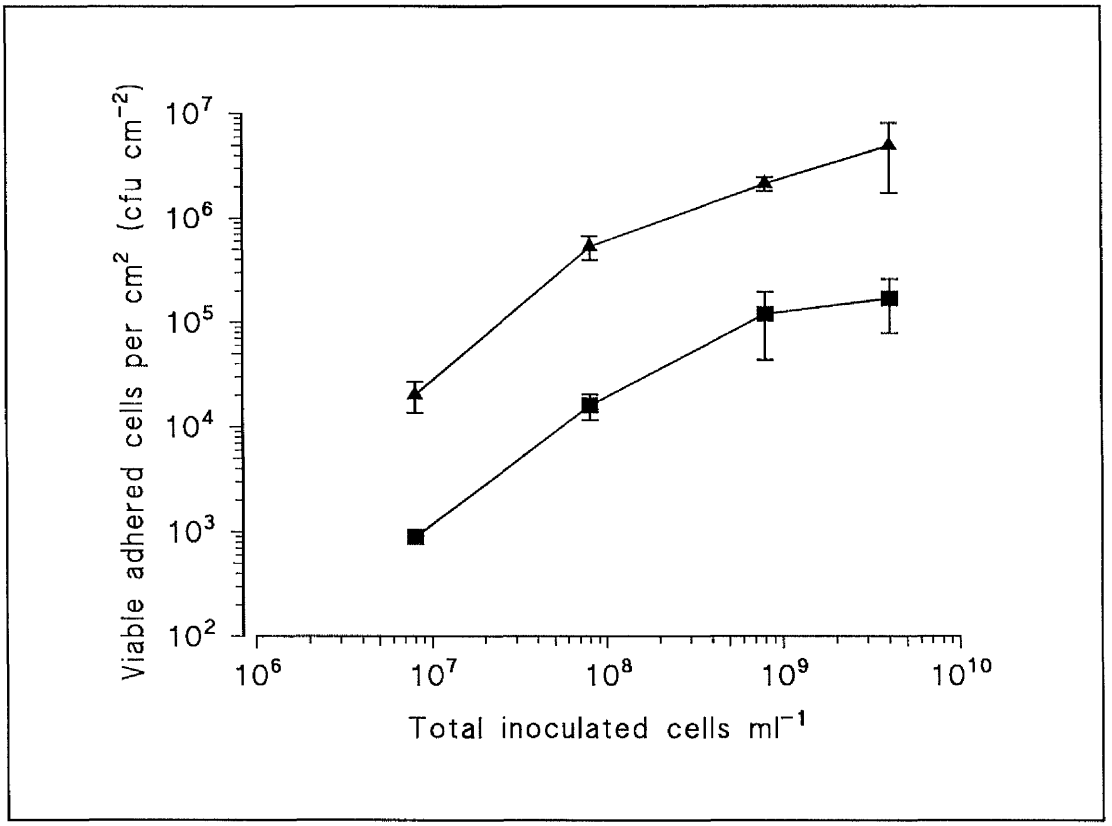
approximately 25, from 0 to 1 d. This was comparable to the 30 fold increase measured on lettuce tissue in Section 3.2.1 where cells were suspended in both deionized water and 1/4 strength Ringer's solution to an inoculum concentration equal to that of inoculum B.

Binding isotherms of adhesion data at both 0 and 1 d are shown in Fig. 3.5. The number of adhered cells at each day (calculated as viable cells per cm^2 of inoculated leaf tissue) is plotted against the total number of cells (per ml) in the inoculum at 0 d. Saturation of available adhesion sites on the substratum is reached when the number of adhered cells is insensitive to an increase in the number cells in suspension. From both curves it can be seen that saturation occurred above an inoculum concentration of 8.0 x 10^7 (Inoculum C) and below or at, a concentration of 8.0 x 10^8 cells ml^{-1} (Inoculum B). This conclusion can be drawn because the number of adhered cells did not increase significantly above the latter cell concentration, where the curves can be seen to plateau, but did increase significantly between the two inoculum concentrations. Because both 0 and 1 d binding isotherms begin to plateau between the same cell concentrations, this would suggest that saturation of adhesion sites on the surface is not time dependent. The number of cells which become adhered to the substratum for each inoculum concentration is, however, time dependent.

Because, for each inoculum concentration used, the numbers of adhered cells increased from 0 to 1 d by a factor of approximately 25, the two binding isotherms appear almost parallel. The reason for this standard increase from 0 to 1 d may be attributed to a steady rate of adhesion over time which is independent of inoculum concentration. The rate of adhesion on lettuce tissue also appears to be independent of cell-suspending medium, as has been indicated by the 30 fold increase in adherent cells from 0 to 1 d when cells were suspended in either deionized water or 1/4 strength Ringer's solution (Section 3.2.1).

The steepest section on each binding isotherm, correlates to the highest percentage adhesion level at that day. At 1 d, inoculum C produced a percentage adhesion level of 0.660%, S.E. = 0.172. This was significantly higher than the percentage adhesion levels produced by the other three inocula (Table 3.4). From the

Figure 3.5 Binding isotherms to lettuce leaf tissue for *L. monocytogenes* CRA 433 cells suspended in 1/4 strength Ringer's solution.



■ = 0 d after inoculation.

▲ = 1 d after inoculation.

Numbers of adhered cells, at both 0 and 1 d, are plotted against total inoculated cells at 0 d.

Data are calculated as the number of viable adhered cells recovered per cm^2 of inoculated leaf tissue.

Values are the means of seven bacterial counts taken from each of seven replicate lettuce discs. Bars shown are standard errors of the mean.

Table 3.4 Effect of cell population level on the adhesion of *L.monocytogenes* to lettuce leaf tissue over a 1 d incubation period at 10°C.

Inoculum Concentration Total cells ml^{-1}	% Adhesion Day 0	Day 1
A) 4.0×10^9	0.003 ± 0.002^a	0.123 ± 0.079^a
B) 8.0×10^8	0.015 ± 0.009^b	0.267 ± 0.040^a
C) 8.0×10^7	0.019 ± 0.005^b	0.660 ± 0.172^b
D) 8.0×10^6	0.011 ± 0.003^b	0.252 ± 0.083^a

Data represent the number of viable adhering cells recovered (per cm^2) at 0 d and 1 d after inoculation expressed as a percentage of the total cells (per ml) inoculated onto leaf tissue at 0 d.

Values are the average of seven replicate lettuce discs $\pm$ standard errors of the mean.

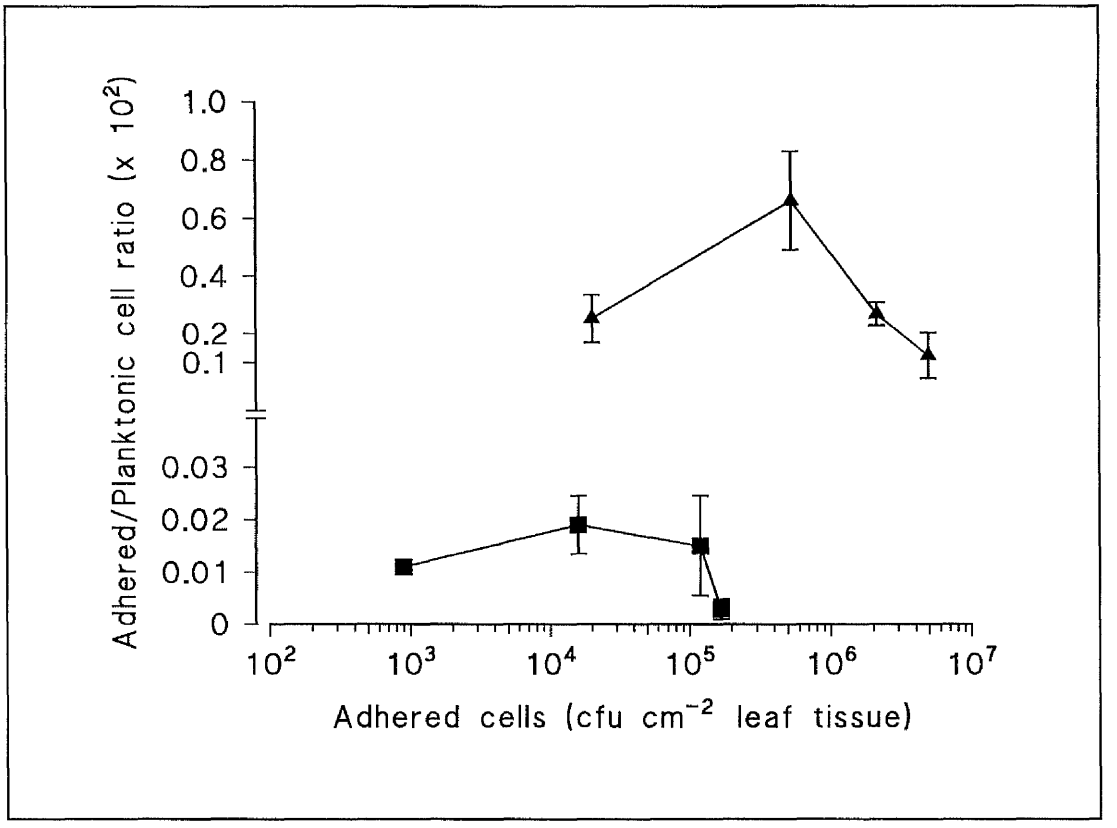
Values in the same column which are followed by the same letter are not significantly different to each other ($p > 0.05$).

binding isotherm for 1 d adhesion data, it is apparent that if the percentage adhesion levels could be increased above 0.66%, this would occur at an inoculum concentration between that of C and D, as this section of the isotherm has the steepest gradient. However, because as stated above, a standard rate of adhesion from 0 to 1 d appears to exist; it appears unlikely that an adhesion level much higher than 0.66% would occur under this regime.

In addition, if the number of adhering cells recovered per disc at 1 d for inoculum C is expressed as the 'true' percentage of total viable cells recovered; this is equal to 3.69%, S.E. = 0.46. This was significantly higher than the maximum 1.93% adhesion level recorded in Section 3.2.1, but still indicates that only a very small proportion of the actual inoculated cells became adhered to the leaf surface following incubation.

A Scatchard plot of adhesion data is presented in Fig. 3.6. The ratio of adhered to planktonic cells is plotted against the number of adhered cells at both 0 and 1 d. The most characteristic feature of both curves is the positive upward slope, the adhered/planktonic ratio increasing with the binding of more cells up to an intermediate adhering cell concentration. These positive slopes indicate that at low adhering cell densities, positive cooperativity exists between adhering cells (i.e. the filling of one site enhances the filling of additional sites). This suggests that cooperativity between *L. monocytogenes* cells promotes their initial adhesion to lettuce leaf tissue. Above intermediate binding cell densities both curves exhibit a negative downward slope, the adhered/planktonic ratio decreasing with the continual binding of more cells. Both negative slopes appear to be continuously changing. This would imply that negative cooperativity exists above intermediate cell densities, and suggest that the population of sites on the substratum is heterogenous or that when one site is filled it inhibits the filling of additional sites. Negative cooperativity does not reflect competition between adherent cells, rather, it exists when the binding of a site by one cell results in the diminution in the affinity of another site for another cell.

Figure 3.6 Scatchard plot of adhered *L. monocytogenes* cells on lettuce leaf tissue at both 0 and 1 d following inoculation.



■ = 0 d after inoculation.

▲ = 1 d after inoculation.

The adhering to planktonic cell ratio is plotted against the number of adhered cells recovered per cm^2 of inoculated leaf tissue at 0 and 1 d.

Data are calculated as the number of viable adhered cells recovered per cm^2 of leaf tissue divided by the total number of cells ml^{-1} in planktonic suspension.

Values are the means of seven bacterial counts taken from each of seven replicate lettuce discs. Bars shown are standard errors of the mean.

3.2.4 Importance of monovalent and divalent electrolytes in the adhesion of *L. monocytogenes* to Dutch White cabbage leaf tissue at 10°C.

3.2.4.i Cabbage leaf tissue.

L. monocytogenes CRA 433 was grown in TSB medium at 10°C until early-stationary phase. One cell batch was collected by centrifugation, washed x3 and divided into four equal volumes. Each volume was resuspended to a total cell concentration of 8.0×10^8 cells ml^{-1} in either deionized water, 1/4 strength Ringer's solution or 40 mM solutions of NaCl or $MgCl_2.6H_2O$.

Abaxial surfaces of cabbage leaf discs were inoculated (2.0×10^7 total cells per disc) with either of the four cell inocula and incubated at 10°C. Planktonic and adhered populations were recovered at 0, 1, and 3 d after inoculation as described previously.

The number of planktonic and adhering cells recovered over the 3 d incubation period are shown in Fig. 3.7.a & b respectively.

At 0 d, numbers of adhering cells were not significantly different between the three salt solutions (Fig. 3.7.b). These adhesion levels were all significantly higher than for cells suspended in deionized water ($p < 0.05$, Multiple Range test). For all four cell-suspending media, adhesion increased significantly ($p < 0.05$, Multiple Range test) from 0 d to a maximum level at 1 d. These increases were by factors of 12 (deionized water), 235 (NaCl), 280 (1/4 strength Ringer's) and 315 ($MgCl_2.6H_2O$). Adhesion of cells suspended in deionized water was significantly lower ($p < 0.05$), at all three sampling times, than adhesion levels for cells suspended in either of the three salt solutions. At both 1 and 3 d the adhesion level for $MgCl_2.6H_2O$ was significantly higher ($p < 0.05$) than that of 1/4 strength Ringer's solution, which in turn was significantly higher than the adhesion level for cells suspended in NaCl.

The maximum adhesion levels for the four cell-suspending media, when calculated as 'true' percentage adhesion levels are equal to 0.93% (deionized water), 13.54% (NaCl), 20.44% (1/4 strength Ringers solution) and 28.74% ($MgCl_2.6H_2O$). This indicates that very high proportions of the inoculated cells became adhered to the leaf tissue when suspended in either of the three salt solutions and that this proportion

Figure 3.7 Effect of cell-suspending medium on the numbers of planktonic and adhering *L. monocytogenes* cells recovered from inoculated abaxial cabbage leaf surfaces over a 3 d incubation period at 10°C.

Cell-suspending media.

▲ = deionized water.

■ = 1/4 strength Ringer's solution.

▼ = 40 mM NaCl.

♦ = 40 mM $MgCl_2.6H_20$.

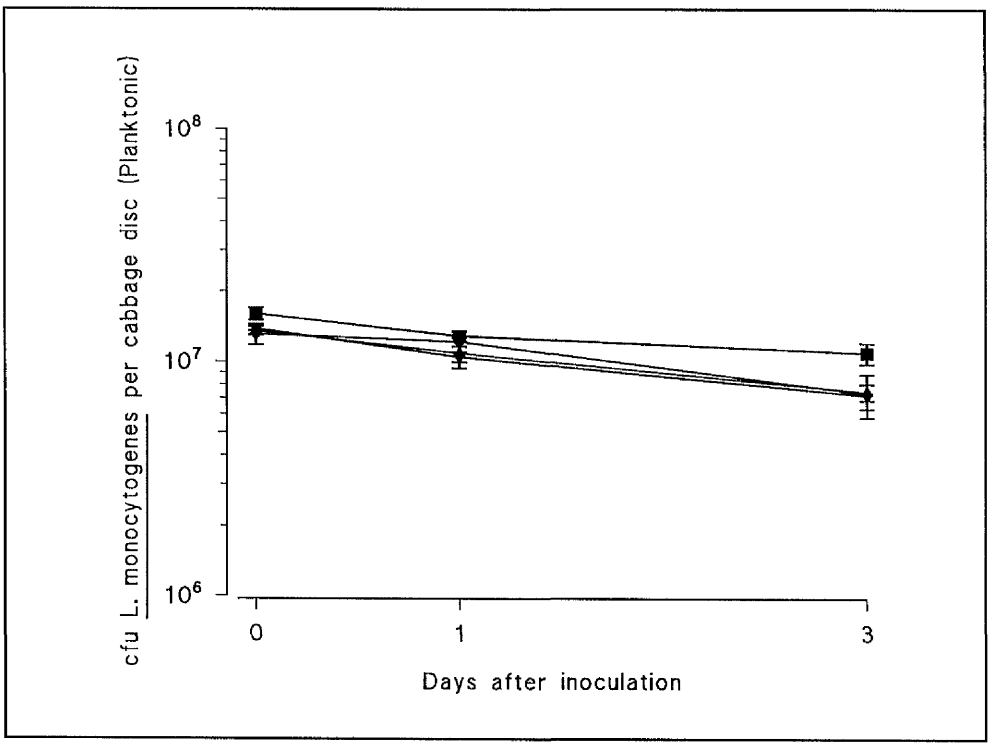
a) Planktonic population (Number of *L. monocytogenes* cells recovered in leaf washing suspensions).

b) Adhering population (Number of *L. monocytogenes* cells recovered from the leaf macerate).

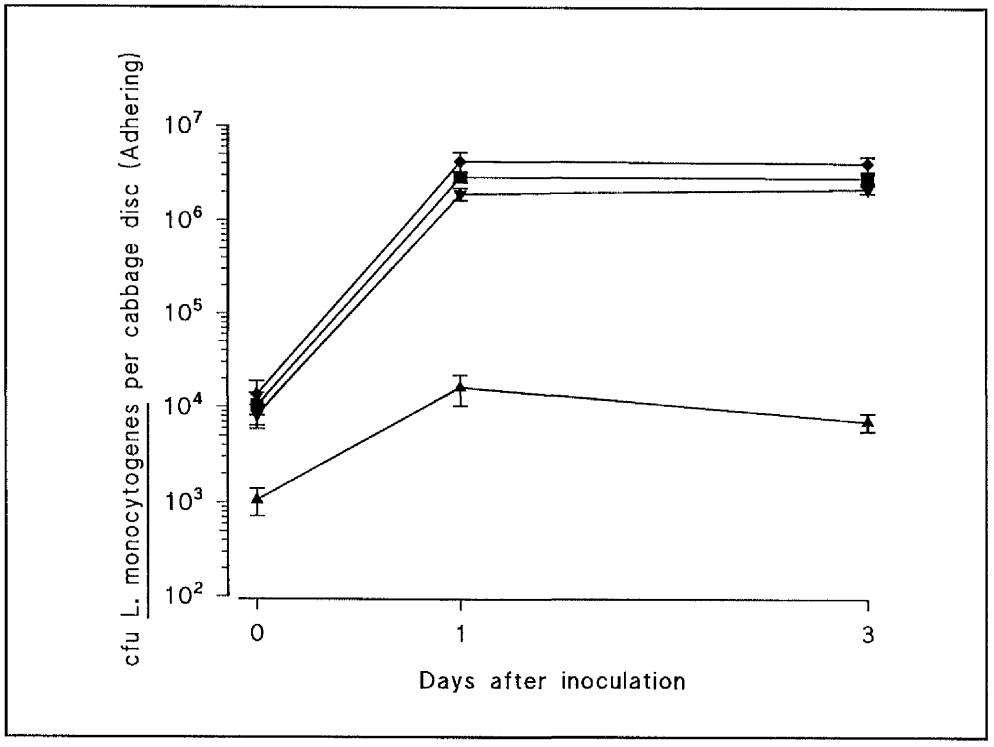
Data are calculated as the number of cells recovered per disc for four bacterial cell-suspending media.

Values are the means of seven bacterial counts taken from each of seven replicate lettuce discs. Bars shown are standard errors of the mean.

a)


cfu L. monocytogenes per cabbage disc (Planktonic)
10^8
10^7
10^6
0
1
3
Days after inoculation

b)


cfu L. monocytogenes per cabbage disc (Adhering)
10^7
10^6
10^5
10^4
10^3
10^2
0
1
3
Days after inoculation

was much lower for cells suspended in deionized water.

At each sampling time, the number of planktonic cells was not significantly different ($p > 0.1$, ANOVA) between the four cell-suspending treatments (Fig. 3.7.a). All planktonic cell populations did, however, decrease significantly ($p < 0.05$) over the three day incubation period. For cells suspended in either NaCl, $MgCl_2.6H_2O$ or 1/4 strength Ringer's solution, up to 70% of the decrease in planktonic cells, from 0 to 3 d, could be accounted for by considering the increase in their adherent populations over this time period. When total cell populations (i.e. the sums of the adhered and planktonic cells) were calculated for the three media; these were found to be not significantly different ($p > 0.1$) from 0 to 3 d. This implies that the fall in planktonic cells recovered for the three media was due purely to a high proportion of inoculated cells becoming adhered. For cells suspended in deionized water, however, only 1% of the decrease in planktonic populations from 0 to 3 d could be accounted for by considering adhering cells. Consequently, the total cell population for cells suspended in deionized water was significantly lower at 3 d than at 0 d. This implies that the presence of salts in the cell-suspending medium is important in maintaining the level of planktonic cell viability on cabbage tissue.

In addition to cabbage discs inoculated with *L. monocytogenes*, negative control discs were also prepared to assess the numbers of naturally occurring bacterial flora present on the cabbage tissue. The control discs, which had been inoculated with sterile 1/4 strength Ringer's solution and incubated at 10°C, did not yield detectable numbers of bacterial colonies on LSA media (less than 78 cfu per cabbage disc) at either 0, 1 or 3 d after incubation. Therefore, only low (undetectable) numbers of naturally occurring bacterial flora, which potentially could have been confused with *L. monocytogenes* colonies on LSA, were present on the leaf tissue. These were unlikely to have affected the accuracy of the bacterial count of *L. monocytogenes*.

3.2.4.i.a Comparison between total and viable adhered cells detected on cabbage tissue

In Table 3.5. percentage adhesion levels to cabbage tissue at 0, 1 and 3 d are

presented for the four cell-suspending media. Percentage adhesion levels at 3 d, for both deionized water and 1/4 strength Ringer's solution, may be compared to the percentage adhesion levels described in Section 3.1.2.i.b. These data were obtained from total bacterial counts of adhered cells on cabbage leaf tissue using SEM adhesion analysis. This comparison is justified because inoculum concentrations for the two experiments were equal (8.0×10^8 total cells ml^{-1}). From this comparison, an excellent correlation in adhesion levels within the two cell-suspending media existed. Adhesion levels for deionized water 0.009% (from SEM adhesion analysis) and 0.011%, S.E.= 0.002 (from leaf macerate counts) were not significantly different ($p > 0.1$, Student's t-test). Similarly, for 1/4 strength Ringer's solution, percentage adhesion levels of 4.22% (from SEM adhesion analysis) and 4.42%, S.E. = 0.487 (from leaf macerate counts) were also not significantly different ($p > 0.1$). It should be remembered that for SEM adhesion analysis data, percentage adhesion levels were calculated from total numbers of adhering cells. In contrast, percentage adhesion levels from leaf macerate counts were calculated from viable adhering cells. Consequently, because an excellent correlation was observed between viable and total percentage adhesion levels, this suggests that all of the adhering *L. monocytogenes* cells observed in SEM images of cabbage tissue (Section 3.1.2) were in fact viable. This applied for both cell-suspending media.

3.2.4.ii Comparison of percentage adhesion levels on cabbage and lettuce leaf tissue.

Table 3.6 summarises selected percentage adhesion levels calculated for lettuce leaf tissue in Sections 3.2.1, 3.2.2 and 3.2.3. These adhesion levels were all for *L. monocytogenes* CRA 433 cells which had been grown in TSB medium and resuspended to a total cell concentration of 8.0×10^8 cells ml^{-1}. Where measured, data is presented for cells suspended in both deionized water and 1/4 strength Ringer's solution. These percentage adhesion levels may be directly compared to those presented for cabbage leaf tissue in Table 3.5 (calculated in Section 3.2.4.i); as these were obtained under identical cell-growth and resuspending conditions.

From the comparison of data in Tables 3.5 and 3.6, it is firstly apparent that for

Table 3.5 Effect of cell-suspending medium on the adhesion of *L. monocytogenes* to cabbage leaf tissue over a 3 d incubation period at 10°C.

Data represent the number of viable adhering cells recovered (per cm^2) at 0, 1 and 3 d after inoculation expressed as a percentage of the total cells (per ml) inoculated onto leaf tissue at 0 d.

Values are the means of seven bacterial counts taken from seven replicate cabbage discs ± standard errors of the mean.

Values followed by the same letter are not significantly different from each other ($p > 0.05$).

Table 3.6 Effect of cell-suspending medium on the adhesion to lettuce leaf tissue at 10°C for *L. monocytogenes* cells grown in TSB and resuspended to a total cell concentration of 8.0×10^8 cells ml^{-1}.

Data presented were collated from percentage adhesion levels calculated for lettuce leaf tissue in Sections 3.2.1, 3.2.2, and 3.2.3. These adhesion levels have previously been presented in Tables 3.1, 3.2 and 3.4 respectively. All data were obtained from leaf tissue which had been inoculated with *L. monocytogenes* CRA 433 cells grown in TSB medium and resuspended to a total cell concentration of 8.0×10^8 cells ml^{-1} in either deionised water or 1/4 strength Ringer's solution.

Data represent the number of viable adhering cells recovered (per cm^2) at 0, 1, and 3 d after inoculation expressed as a percentage of the total cells (per ml) inoculated onto leaf tissue at 0 d. ND = not determined.

Values are the means of seven bacterial counts taken from either five ♦, six # or seven * replicate lettuce discs ± standard errors of the mean.

Values followed by the same letter are not significantly different from each other ($p > 0.05$).

Note: Values in Tables 3.5 and 3.6 which are followed by the same letter are also not significantly different from each other ($p > 0.05$).

Table 3.5

Cell Suspending Medium	% Adhesion		
	Day 0	Day 1	Day 3
deionized water	0.002 ± 0.0005^{a}	0.020 ± 0.008^{e}	0.011 ± 0.002^{e}
40 mM NaCl	0.013 ± 0.003^{b}	2.989 ± 0.435^{d}	3.387 ± 0.336^{d}
1/4 Ringer's solution	0.016 ± 0.006^{b}	4.513 ± 0.544^{e}	4.423 ± 0.487^{e}
40 mM $MgCl_2.6H_2O$	0.021 ± 0.008^{b}	6.619 ± 1.557^{f}	6.340 ± 1.134^{f}

Table 3.6

Cell Suspending Medium (Section Data Obtained)	% Adhesion		
	Day 0	Day 1	Day 3
deionized water (Section 3.2.1)	$0.002 \pm 0.000^{\blacklozenge a}$	$0.052 \pm 0.042^{\#c}$	0.085 ± 0.083^{ch}
1/4 Ringer's solution (Section 3.2.1)	$0.009 \pm 0.003^{\blacklozenge b}$	$0.330 \pm 0.213^{\#h}$	0.069 ± 0.050^{ch}
1/4 Ringer's solution (Section 3.2.2)	$0.031 \pm 0.015^{\#bc}$	$0.268 \pm 0.071^{\blacklozenge h}$	$0.444 \pm 0.112^{\#h}$
1/4 Ringer's solution (Section 3.2.3)	$0.015 \pm 0.009^{*b}$	$0.267 \pm 0.040^{*h}$	ND

cells suspended in deionized water, percentage adhesion levels at 0 d on cabbage and lettuce tissue were identical (0.002%). Adhesion levels in deionized water increased significantly from 0 to 1 d for both cabbage and lettuce; adhesion levels at 1 d were also not significantly different between the two tissue types. Consequently it may be implied that no significant differences could be detected in the adhesion trend for cabbage and lettuce leaf tissue when cells were suspended in deionized water. For cells suspended in 1/4 strength Ringer's solution, the percentage adhesion levels at 0 d for cabbage and lettuce tissue (approximately 0.02%) were also not significantly different. However, by 1 d adhesion levels for cabbage tissue had increased, 280 fold from 0 d, to a percentage adhesion level of 4.513%, whereas adhesion levels for lettuce tissue had only increased by a maximum factor of 37. Consequently, adhesion levels on cabbage tissue at 1 d were significantly higher ($p < 0.05$) than adhesion levels on lettuce tissue. This significant difference was also observed at 3 d.

3.3 Cell surface characteristics of *Listeria* species.

Two cell-surface characteristics of *Listeria* were investigated. These were; the presence of filamentous surface appendages (Section 3.3.1), and the expression of cell surface hydrophobicity (Section 3.3.2).

3.3.1 Examination of negatively stained of *Listeria* species by Transmission electron microscopy.

Using the negative staining technique outlined in Section 2.6, *Listeria* species including, *L. monocytogenes* CRA 433, *L. innocua*, *L. ivanovii*, *L. welshimeri* and *L. seeligeri* were examined for the presence of cell-surface appendages. All species produced several peritrichous flagella, up to 6 μm in length, after both a 48 and 72 h incubation period in Tryptone Soy broth (TSB), Brain Heart Infusion broth (BHI) or on Tryptone Soy agar (TSA) at 10°C (Plate 3.7). In addition, *L. monocytogenes* CRA 433 was shown to produce such structures after 24 h incubation in TSB, BHI or on TSA at 25°C.

When *L. monocytogenes* CRA 433 was incubated in TSB at 25°C for an extended

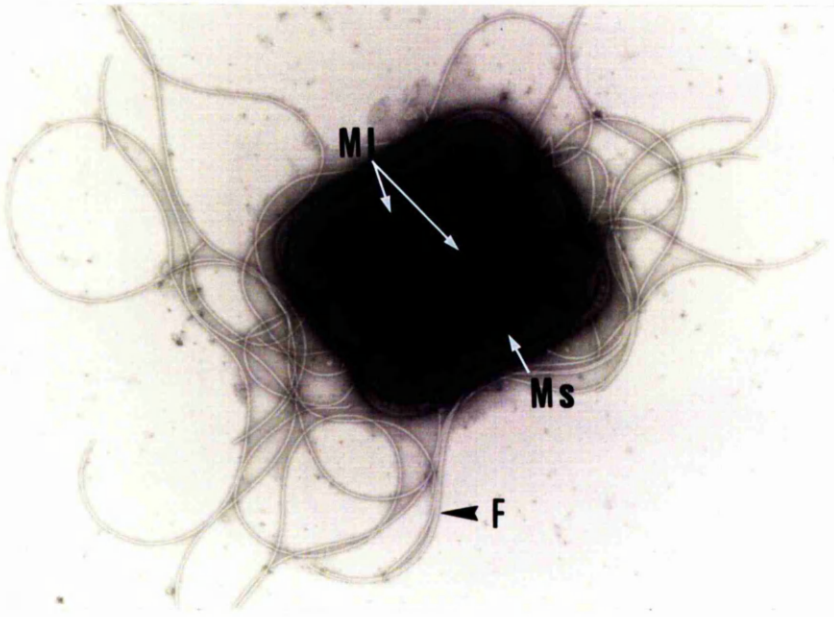
period of 7 d, cells approximately 15.0 μm in length were produced in addition to cells of more normal length 0.5-1.5 μm (Plate 3.8). All cell types had peritrichous flagella.

Distinctive membranous structures called mesosomes were seen in all *Listeria* cells examined. For cells not undergoing division, mesosomes were relatively small (up to approximately 1/2 the width of the cell) and occurred randomly and at a relatively high frequency throughout the length of the cell body (Plates 3.7, 3.8 & 3.9). For cells undergoing division, in addition to a relatively low frequency of small mesosomes, a single large mesosome which spanned the width of the cell was also observed in the area of septum formation (Plates 3.7. & 3.8). During the latter stages of cell division, this large mesosome was separated by the newly formed cell wall and was then incorporated into the two individual daughter cells.

Under each of the growth regimes described above, neither *L. monocytogenes* CRA 433, *L. innocua*, *L. ivanovii*, *L. welshimeri* or *L. seeligeri* were shown to possess non-flagellar filamentous appendages such as fimbriae or fibrils (Plate 3.9).

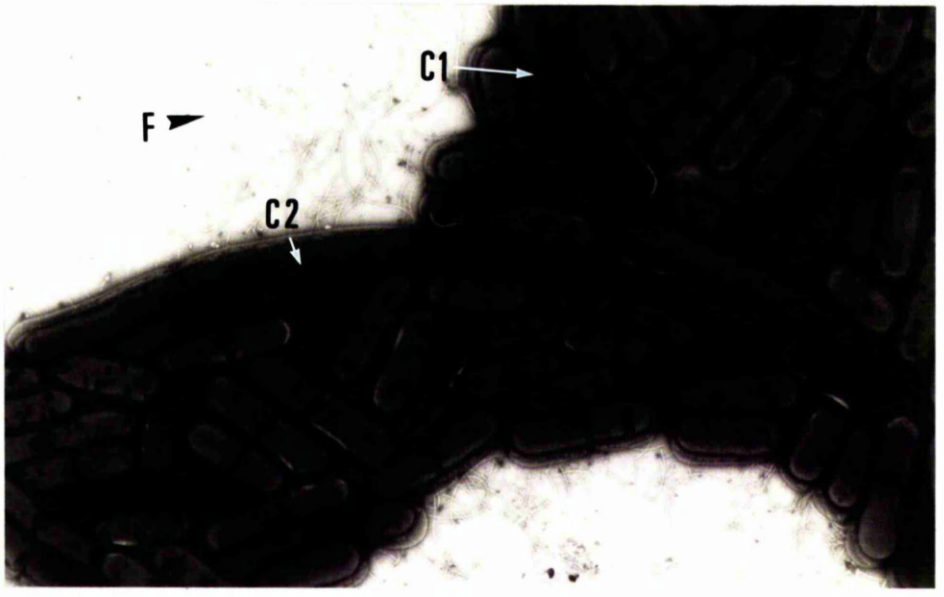
The positive control organism *Streptococcus sanguis* (CN3410) was also negatively stained following overnight incubation in BHI at 37°C. Handley *et al* (1984) showed that, under this growth regime, *S. sanguis* (CN3410) had a tuft consisting of short and long fibrils as well as peritrichous fibrils visible on its surface. All of these structures were also observed in this study (Plate 3.10).

Plate 3.7 Negative staining of *L. monocytogenes* CRA 433 cells grown in Tryptone Soy broth at 10°C for 72 h.



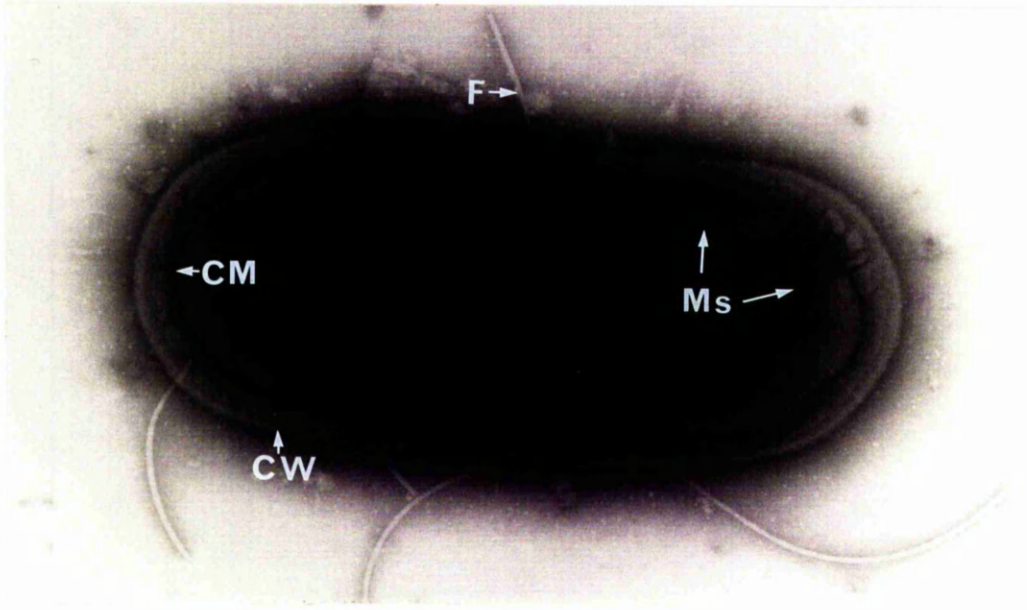
A clump of three cells is shown. Flagella (F) are ≤ 6 μm in length. Large mesosomes (Ml) are present in the area of septum formation for the two dividing cells. Only small mesosomes (Ms) are visible for the un-dividing cell (x 21600 magnification).

Plate 3.8 Negative staining of *L. monocytogenes* CRA 433 cells grown in Tryptone Soy broth at 25°C for 7 d.



Two filamentous cells (C1 & C2) with flagella (F) are indicated (x 10200 magnification).

Plate 3.9 Negative staining of *L. monocytogenes* CRA 433 grown in Tryptone Soy broth at 10°C for 72 h.



One un-dividing cell with numerous small mesosomes (Ms) which extend from the cell membrane (CM) is shown. The cell wall (CW) and flagella (F) are also indicated. Non-flagellar filamentous appendages were not present (x 54000 magnification).

Plate 3.10 Negative staining of *Streptococcus sanguis* CN3410 grown in Brain Heart Infusion broth at 37°C for 24 h.

The isolate carries a tuft (T) which consists of short (S) and long (L) fibrils. Peritrichous fibrils (PF) and mesosomes (M) are also visible (x 47000 magnification).

3.3.2 Cell surface hydrophobicity of *Listeria* species.

Hydrophobic characteristics of *Listeria* cells were investigated using three separate protocols (each protocol is detailed in Section 2.9). Results of these three protocols are presented in this section. Firstly, the bacterial adhesion to hydrocarbons (BATH) partition assay, was used to measure the percentage of hydrophobic *Listeria* cells within suspension (results presented in Section 3.3.2.i). Secondly, a modification of the partition assay was used in an attempt to produce a predominantly hydrophobic population of *L. monocytogenes* cells (results presented in Section 3.3.2.ii). Thirdly, colloidal gold was used to label hydrophobic sites on the surface of *L. monocytogenes* cells (results presented in Section 3.3.2.iii).

3.3.2.i Measurement of cell surface hydrophobicity of *Listeria* species using bacterial adhesion to hydrocarbons (BATH test).

The optimised BATH test of Rosenberg *et al.* (1984a), which is outlined in Section 2.9.1, was used in four experiments described in Section 3.3.2.i.a-d. Percentage hydrophobicity values represent the proportion of bacterial cells in original cell suspensions which partitioned into hydrophobic n-hexadecane following vortexing. These cells, therefore, are those which express a net hydrophobic nature.

3.3.2.i.a Effect of growth temperature on the hydrophobicity of five *Listeria* species following incubation in TSB and resuspension in deionized water.

L. monocytogenes CRA 433, *L. innocua*, *L. ivanovii*, *L. seeligeri* and *L. welshimeri* cells were grown in Tryptone Soy broth (TSB) at 10 and 25°C until early-stationary growth phase. All cells were resuspended in deionized water for hydrophobicity measurement.

Table 3.7 shows that the percentage hydrophobicity values of below 4%, were not significantly different between any of the five *Listeria* species tested ($p > 0.05$, ANOVA). In addition, no significant differences in hydrophobicity were found between cells grown at 10 and 25°C.

Table 3.7 Cell surface hydrophobicity of *Listeria* species suspended in deionized water following incubation in TSB at either 10°C or 25°C.

Listeria species	Incubation temperature	% Hydrophobicity
L. monocytogenes	10°C	2.99 ± 0.34[a]
L. innocua	10°C	3.35 ± 0.42[a]
L. seeligeri	10°C	3.50 ± 0.38[a]
L. ivanovii	10°C	3.59 ± 0.25[a]
L. welshimeri	10°C	3.79 ± 0.33[a]
L. monocytogenes	25°C	3.18 ± 0.27[a]
L. innocua	25°C	2.93 ± 0.33[a]
L. seeligeri	25°C	3.70 ± 0.24[a]
L. ivanovii	25°C	3.98 ± 0.35[a]
L. welshimeri	25°C	3.93 ± 0.36[a]

Data represent the mean percentage of cells in original suspensions which partitioned into the hydrophobic n-hexadecane phase following vortexing.

Values are the means of ten replicate samples taken from one cell suspension ± standard errors of the mean.

Results followed by the same letter are not significantly different from each other ($p > 0.05$, ANOVA).

3.3.2.i.b Hydrophobicity of four *L. monocytogenes* isolates when suspended in deionized water and 1/4 strength Ringer's solution.

L. monocytogenes strains CRA 433 4b, CRA 5246 4b, ATCC 23074 4b and F139 1/2a were grown in TSB at 10°C until early-stationary phase. Cells were resuspended in either deionized water or 1/4 strength Ringer's solution for hydrophobicity measurement.

Percentage hydrophobicity values are presented in Table 3.8. When suspended in deionized water, no significant differences in hydrophobicity ($p > 0.05$, ANOVA) were shown between the four *L. monocytogenes* isolates. Hydrophobicity values of between 1 and 3% were considered to be very low. For each individual isolate, hydrophobicity increased significantly ($p < 0.05$, Student's t-test) when cells were suspended in 1/4 strength Ringer's solution compared to deionized water. This increase was less apparent (but still significant) for *L. monocytogenes* F139 1/2a compared to the remaining three 4b isolates. In 1/4 strength Ringer's solution, isolates CRA 433 4b and CRA 5246 4b had approximately 9.3% of hydrophobic cells. This was significantly higher ($p < 0.05$, Multiple Range test) than isolate ATCC 23074 4b (7.7%), which in turn was significantly higher than isolate F139 1/2a (3.37%).

3.3.2.i.c Effect of growth phase on the hydrophobicity of *L. monocytogenes* CRA 433 cells suspended in 1/4 strength Ringer's solution.

L. monocytogenes CRA 433 cells were grown in TSB at 10°C until mid-exponential, early-stationary and late-stationary growth phases as described in Section 2.4.2. Cells were resuspended in 1/4 strength Ringer's solution for hydrophobicity measurement and the experiment repeated in triplicate using three separate cell batches for each growth phase.

Percentage hydrophobicity values for each of the three replicate experiments are presented in Table 3.9. No significant differences ($p > 0.05$, ANOVA) were shown between any of the hydrophobicity values calculated. The data obtained are obviously quite limited but they do indicate that good inter-batch reproducibility existed for this optimised assay system.

Table 3.8 Cell surface hydrophobicity of four *L. monocytogenes* isolates suspended in deionized water and 1/4 strength Ringer's solution.

L. monocytogenes isolate	Cell suspending medium	% Hydrophobicity
CRA 433 4b	deionized water	1.88 ± 0.31[a]
CRA 433 4b	1/4 strength Ringer's	9.26 ± 0.26[c]
CRA 5246 4b	deionized water	2.42 ± 0.33[a]
CRA 5246 4b	1/4 strength Ringer's	9.31 ± 0.46[c]
ATCC 23074 4b	deionized water	1.54 ± 0.49[a]
ATCC 23074 4b	1/4 strength Ringer's	7.70 ± 0.37[d]
F139 1/2a	deionized water	1.92 ± 0.30[a]
F139 1/2a	1/4 strength Ringer's	3.37 ± 0.11[b]

Data represent the mean percentage of cells in the original suspension which partitioned into the hydrophobic n-hexadecane phase following vortexing.

Values are the means of ten replicate samples taken from one cell suspension ± standard errors of the mean.

Results followed by the same letter are not significantly different from each other ($p > 0.05$, Multiple Range test).

Table 3.9 Effect of bacterial growth phase on the hydrophobicity of *L. monocytogenes* CRA 433 cells when resuspended in 1/4 strength Ringer's solution.

Bacterial growth phase	% Hydrophobicity (Experiment 1)	% Hydrophobicity (Experiment 2)	% Hydrophobicity (Experiment 3)
mid-exponential	8.72 ± 0.49[a]	8.65 ± 0.61[a]	9.27 ± 0.61[a]
early-stationary	8.80 ± 0.37[a]	9.82 ± 0.61[a]	9.10 ± 0.42[a]
late-stationary	9.23 ± 0.45[a]	9.48 ± 0.51[a]	9.74 ± 0.59[a]

Data represent the mean percentage of cells in the original suspensions which partitioned into the hydrophobic n-hexadecane phase following vortexing. Data are shown for three replicate experiments.

Values are the means of ten samples from one cell suspension ± standard errors.

Results followed by the same letter are not significantly different from each other ($p > 0.05$, ANOVA).

3.3.2.i.d Hydrophobicity of *L. monocytogenes* CRA 433 cells when suspended in deionised water and different salt solutions.

One batch of *L. monocytogenes* CRA 433 was grown to early-stationary growth phase in TSB at 10°C. Following incubation, the batch was divided into seven equal volumes and each volume resuspended in one of the following solutions: deionized water, 1/4 strength Ringer's solution or 40 mM solutions of either $NaHCO_3$, NaCl, KCl, $CaCl_2$ or $MgCl_2.6H_2O$. Percentage hydrophobicity values for each cell suspension were then determined and are presented in Fig. 3.8.

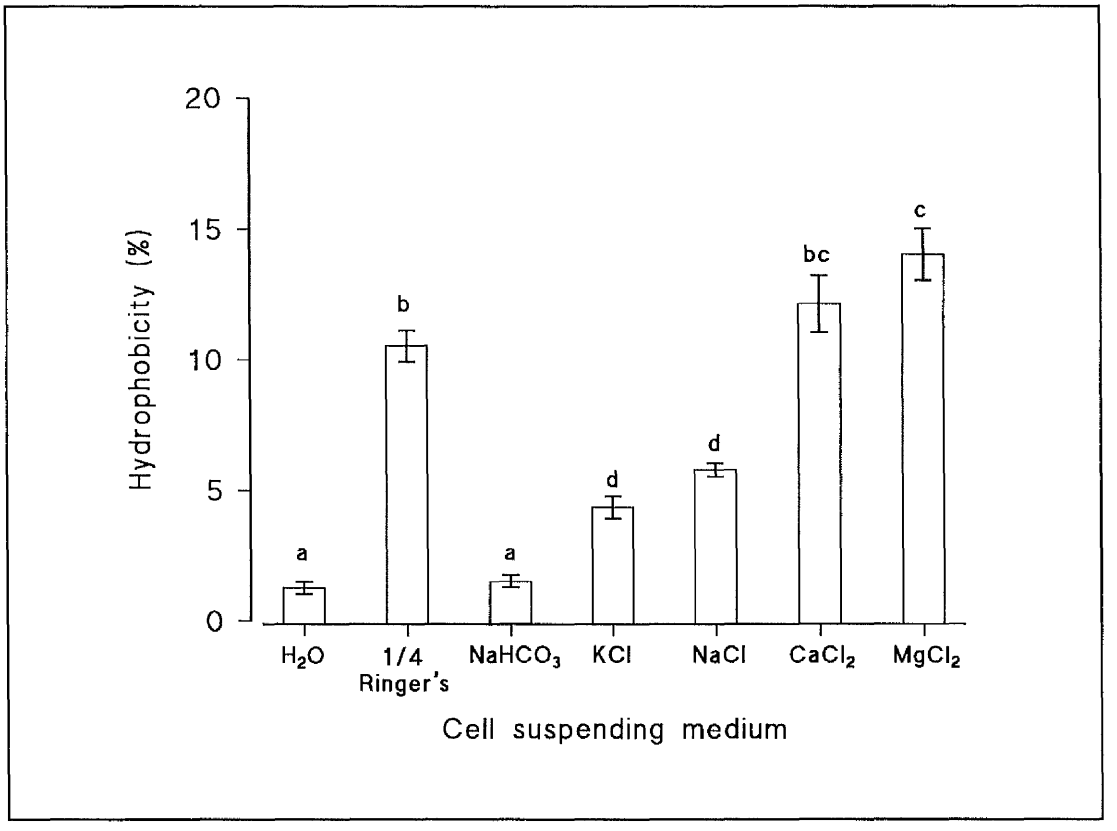
No significant differences in hydrophobicity ($p > 0.05$, Multiple Range test) were found between cells suspended in deionized water (1.26%) compared to $NaHCO_3$ (1.52%), NaCl (5.79%) compared to KCl (4.35%), 1/4 strength Ringer's (10.53%) compared to $CaCl_2$ (12.15%) or between $CaCl_2$ and $MgCl_2.6H_2O$ (14.04%) (Fig. 3.8). Hydrophobicity values for cells suspended in either of the divalent cation solutions $CaCl_2$ or $MgCl_2.6H_2O$ were significantly higher ($p < 0.05$ Multiple Range test) than for cells suspended in the monovalent solutions NaCl or KCl. Similarly, cells suspended in KCl expressed significantly higher levels of hydrophobicity ($p < 0.05$) than cells suspended in either $NaHCO_3$ or deionized water.

3.3.2.ii Attempted production of a predominately hydrophobic population of *L. monocytogenes* cells, by repeated enrichment of hexadecane partitioned cells.

The enrichment BATH test of Cunniffe (1993), detailed in Section 2.9.2 was used in an attempt to produce a population of predominantly hydrophobic *L. monocytogenes* CRA 433 cells. Hydrophobic cells were partitioned from suspension using n-hexadecane, enriched and then again partitioned in an attempt to increase the proportion of hydrophobic cells in the population. This phase separation/enrichment cycles was performed 10 times. The mean and the maximum hydrophobicity values calculated at the end of each cycle for ten replicate samples are presented in Table 3.10.

As shown in Table 3.10, the procedure failed to produce any significant increase in the average percentage hydrophobicity after the ten enrichment cycles. The highest percentage hydrophobicity value calculated was 3.17% after six enrichment procedures.

Figure 3.8 Histogram showing percentage hydrophobicity values calculated for *L. monocytogenes* CRA 433 cells when suspended in deionized water and different 40 mM salt solutions.



Data represent the mean percentage of cells in original cell suspensions which partitioned into the hydrophobic n-hexadecane phase following vortexing.

Values are the means of ten replicate samples taken from one cell batch ± standard errors of the mean (Bars).

Columns labelled with the same letter are not significantly different from each other ($p > 0.05$, Multiple Range test).

Table 3.10 Attempted enrichment of hydrophobic *L. monocytogenes* cells from a predominantly hydrophilic cell population.

Phase Separation/Enrichment cycle	% Hydrophobicity
1	2.78% ± 0.42 (4.90)
2	2.54% ± 0.33 (4.66)
3	2.44% ± 0.25 (3.28)
4	2.07% ± 0.25 (3.08)
5	2.33% ± 0.29 (3.35)
6	3.17% ± 0.49 (4.80)
7	2.76% ± 0.40 (4.81)
8	1.96% ± 0.32 (3.64)
9	2.18% ± 0.37 (4.01)
10	2.25% ± 0.39 (4.63)

Data shown represent the average percentage hydrophobicity values calculated over ten phase separation/enrichment cycles.

Values are the means of ten replicate samples taken from each enriched cell suspension ± standard errors of the mean.

Values in parentheses show the maximum percentage hydrophobicity value calculated from 10 replicate samples. This hydrophobic cell population was used as inoculum for the subsequent phase separation procedure.

3.3.2.iii Labelling of hydrophobic sites on *L. monocytogenes* CRA 433 cells with colloidal gold.

Hydrophobic sites on cells of *L. monocytogenes* CRA 433 were labelled with 10 nm colloidal gold, using the procedures of Handley *et al.* (1991) as outlined in Section 2.9.3. One batch of *L. monocytogenes* CRA 433 cells were labelled following incubation in TSB to early-stationary phase and resuspension in deionized water, 1/4 strength and 1/2 strength Ringer's solution. The distribution and frequency of colloidal gold particles on the cell surface of *L. monocytogenes* was assessed by TEM examination of cells labelled at pH 3.5, 5.2, 7.1 and 9.6.

3.3.2.iii.a Labelling of cells suspended in deionized water.

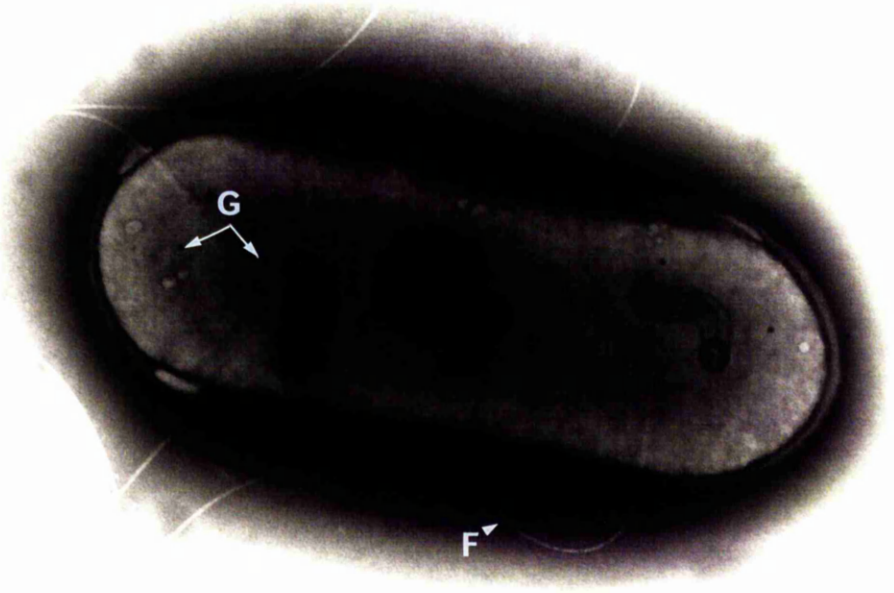
The level of colloidal gold labelling of cells suspended in deionized water was very low. At each pH 3.5, 5.2, 7.1 and 9.6, two hundred *L. monocytogenes* cells were examined for colloidal gold labelling. Of these 200 cells only 6 (pH 3.5), 4 (pH 5.2), 7 (pH 7.1) and 8 (pH 9.6) cells showed any colloidal gold particles associated with them, the highest level of which (9 particles per cell) is shown in Plate 3.11. Of the total 25 labelled cells only four had 6 or more gold particles distributed individually over their cell surface. The remaining 775 cells (96.9%) examined did not have any gold particles associated with their cell surface. In addition, no cells examined showed any labelling of flagella.

The background count of gold labelling in grid areas between *L. monocytogenes* cells was very low. Ten areas per grid of magnification 20 K were sampled and only 3 gold particles were observed.

3.3.2.iii.b Labelling of cells suspended in 1/4 strength Ringer's solution.

Below a pH of 5.2, colloidal gold flocculated in the presence of 1/4 strength Ringer's solution. Labelling of cells below pH 5.2 could therefore not be assessed. At pH 5.2, 7.1 and 9.6, a much higher proportion of cells were labelled with colloidal gold in 1/4 strength Ringer's solution compared to deionized water. Examination of 300 cells (100 per pH value) showed that only 11 (3.6%) had fewer than 6 gold particles

Plate 3.11 Negative staining of *L. monocytogenes* CRA 433 labelled with 10 nm colloidal gold at pH 9.6 in deionised water.



The maximum number of colloidal gold particles (G) associated with a *L. monocytogenes* CRA 433 cell (9 particles over the cell body) is shown. Flagella (F) did not label with colloidal gold (x 82500 magnification).

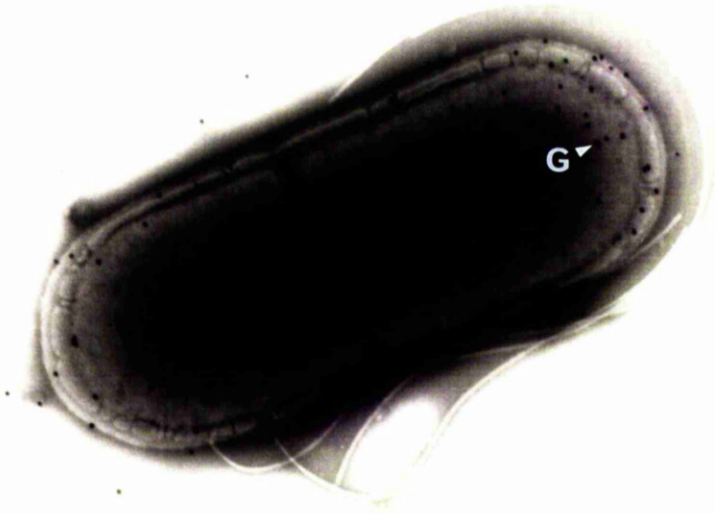
distributed over their cell surface. This compares to 96.9% of cells in deionized water which were un-labelled. The maximum number of gold particles per cell observed was also higher; 80 gold particles (pH 9.6) were present on one cell in the early stages of division (Plate 3.12.a) and 72 gold particles (pH 9.6) on one un-dividing cell (Plate 3.12.b). This is compared to 9 particles per cell (Plate 3.11) seen previously in deionized water. For all cells examined, colloidal gold labelling was limited to the cell surface, individual gold particles distributed randomly over the cell surface, flagella were not labelled at any of the pH values tested.

Of the 100 *L. monocytogenes* cells examined per pH level, the number of gold particles associated with the cell surface varied between 2 to 34 (pH 5.2), 1 to 37 (pH 7.1) and, 2 to 80 (pH 9.6). As described above, at pH 9.6 only 2 cells had above 60 gold particles on their cell surface. In Fig. 3.9.a, b & c (pH values 5.2, 7.1 and 9.6 respectively) a range of 0-60 particles per cell are plotted as 12 sub-divisions, increasing by increments of five gold particles i.e 0-5, 6-10, 11-15 etc., against which the number of cells occurring in each of these 12 ranges is shown. Fig 3.9 demonstrates that for each pH value tested, the overall range of gold labelling in the 100 cells sampled, was normally distributed around a mean labelling level. At pH 5.2, 7.1 and 9.6 these mean levels of labelling were 15.8, 16.1 and 25.0 gold particles per cell respectively. No significant difference between the mean levels of labelling at pH 5.2 and 7.1 was observed ($p > 0.05$ Multiple Range test). At pH 9.6 the mean level of labelling was significantly higher than at pH 5.2 and 7.1 ($p < 0.05$). Plate 3.13 illustrates two *L. monocytogenes* cells each labelling approximate to the mean 25.0 particles per cell level at pH 9.6.

The background count of gold labelling in grid areas between *L. monocytogenes* cells was low. Ten areas per grid of magnification 20 K were sampled and only 11 gold particles were observed. A small number of gold particles, 1 or 2 per cell, were often seen in their immediate vicinity (i.e within a 0.5 μm distance of the cell body) of labelled cells but not associating with either the flagella or the cell body.

Plate 3.12 **Negative stains of *L. monocytogenes* CRA 433 labelled with 10 nm colloidal gold at pH 9.6 in 1/4 strength Ringer's solution.**

a)



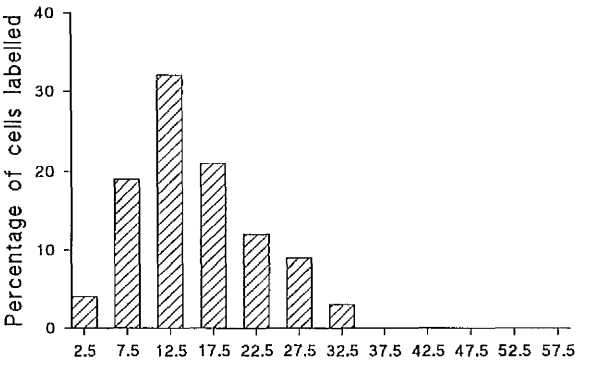
b)

a) The maximum number of colloidal gold particles (G) associated with a dividing cell (80 particles) is shown (x 64000 magnification).

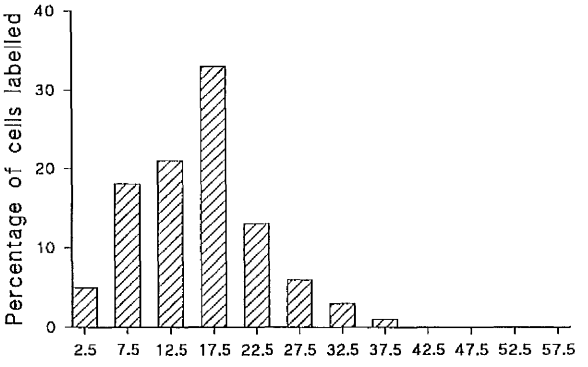
b) The maximum number of colloidal gold particles (G) associated with an un-dividing cell (72 particles) is shown (x 68000 magnification).

Figure 3.9 Frequency distributions of colloidal gold labelling of *L. monocytogenes* cells suspended in 1/4 strength Ringer's solution at pH 5.2, 7.1 and 9.6.

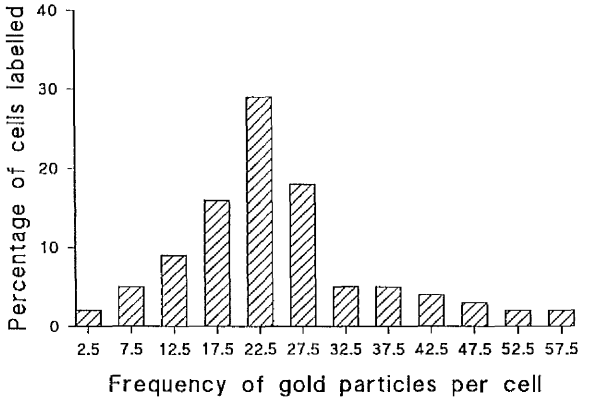
a) pH 5.2



b) pH 7.1



c) pH 9.6



Data for each pH level illustrate the number of colloidal gold particles associated with each of 100 *L. monocytogenes* cells when suspended in 1/4 strength Ringer's solution.

For each pH, four replicate TEM grids were examined and the number of gold particles associated with 25 cells on each grid were counted.

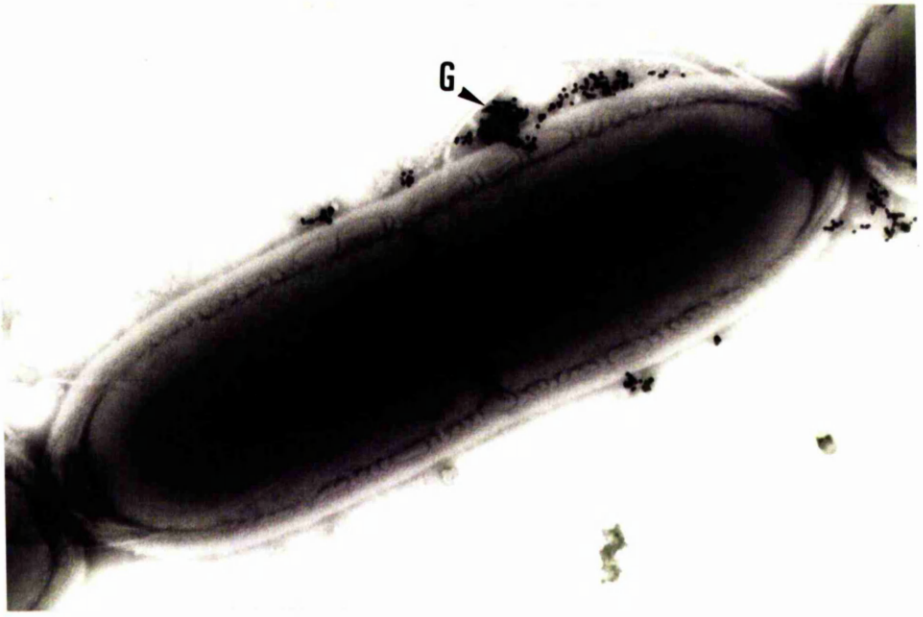
3.3.2.iii.c Labelling of cells suspended in 1/2 strength Ringer's solution.

The addition of 1/2 strength Ringer's solution to colloidal gold at pH 5.2, 7.1 and 9.6 resulted in the colloidal gold particles flocculating in suspension. Flocculation was characterised by a colour change from red to blue of the colloidal gold sol and occurred most rapidly (within a few seconds of mixing) at the lower pH level. Plate 3.14 illustrates a *L. monocytogenes* cell labelled with colloidal gold which had flocculated at pH 9.6. The colloidal gold particles were no longer independent of each other, occurring in groups of up to approximately 50 or 60 gold particles. Flocculation of colloidal gold was also observed in the presence of deionised water and 1/4 strength Ringer's solution at pH levels below 3.3 and 5.2 respectively.

Plate 3.13 **Negative staining of two *L. monocytogenes* CRA 433 cells labelled with 10 nm colloidal gold at pH 9.6 in 1/4 strength Ringer's solution.**

The mean number of colloidal gold particles (G) observed in a population of 100 cells (approximately 25 particles per cell) is shown for two cells (x 59500 magnification).

Plate 3.14 **Negative staining of *L. monocytogenes* CRA 433 labelled with 10 nm colloidal gold at pH 9.6 in 1/2 strength Ringer's solution.**



Flocculated colloidal gold particles (G) are indicated (x 66000 magnification).

3.4 Biophysical characterisation of leaf and model surfaces. Adhesion of *L. monocytogenes* to model surfaces.

Model surfaces used in this study included; glass coverslips [either chloroform washed (CW), flame oxidised (FO) or not treated (NT) from the supplier], cabbage-wax coated glass coverslips (CBW), Spurr resin, acetate, and dental wax [either untreated or plasma-glow discharged (PGD) after production]. Leaf surfaces included cabbage and lettuce tissue. All surfaces were prepared for characterisation as detailed in Section 2.10.1.

Model surfaces were characterised in terms of their surface roughness (Section 3.4.1) and hydrophobicity (Section 3.4.2). The surface roughness of leaf tissue could not be determined because of large form variations (>200 μm) in the vertical depth profiles of each surface caused by leaf venation. Hydrophobicity of leaf surfaces could be determined and results are presented in Section 3.4.2.

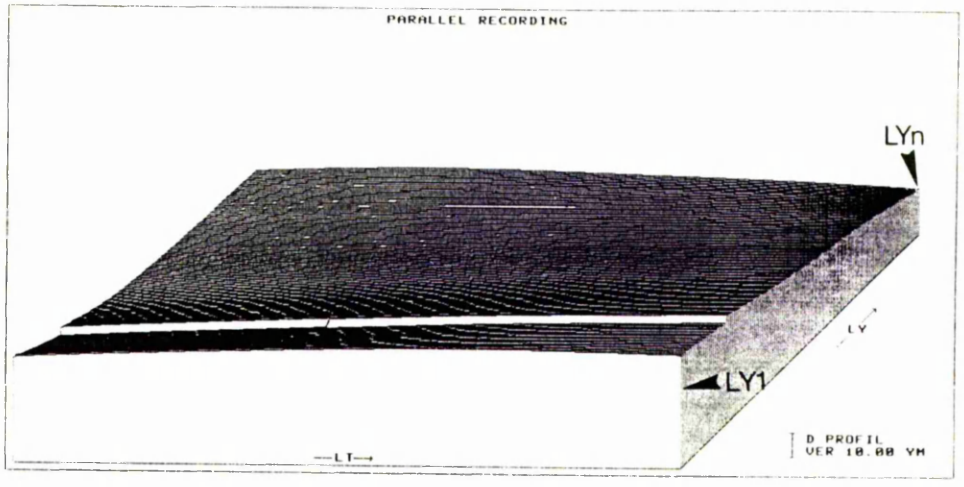
The SEM protocol detailed in Section 2.11, was also used to assess the adhesion of *L. monocytogenes* to the eight model surfaces described above. Results of SEM adhesion analysis for model surfaces is presented in Section 3.4.3. SEM micrographs provide valuable cross reference information which relates to surface roughness data presented in Section 3.4.1.

3.4.1 Surface roughness analysis of model surfaces.

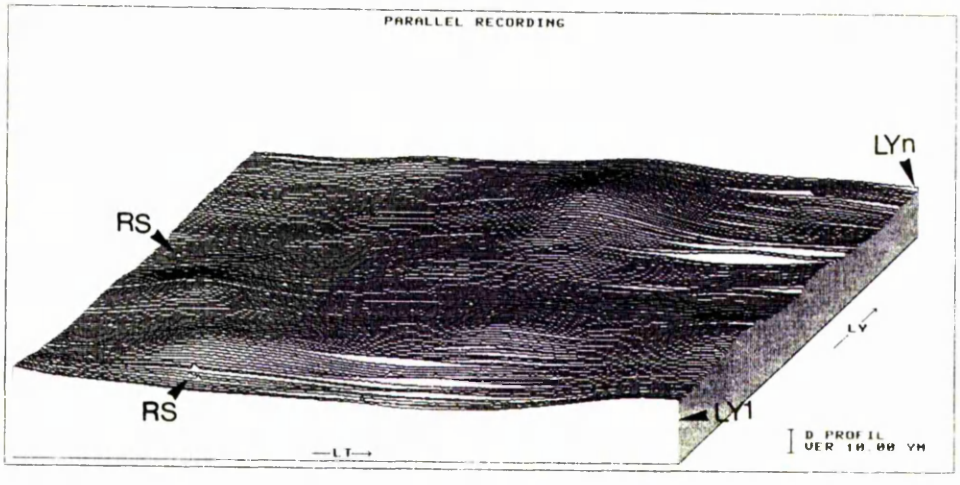
Each of the eight model surfaces described above, was analyzed for surface roughness using the protocol detailed in Section 2.10.2. From one 17.5 x 17.5 mm area of each sample, 129 parallel, directly-traced surface roughness profiles were recorded and plotted collectively, at a vertical depth scale of 10 μm, to produce a parallel recording image. Typical recording images for NT glass coverslip, acetate, Spurr resin and dental wax are illustrated in Fig. 3.10. a-d respectively. For each image the first and last line traces are labelled as LY1 and LYn respectively. The parallel recording images of CW, FO, NT glass and CBW coverslip surfaces were indistinguishable from each other and all resembled the one illustrated for NT glass in Fig 3.10.a. In addition, no distinction could be made between the parallel recording images of dental wax

Figure 3.10 Parallel recording images of four model surfaces illustrated at a vertical depth scale of 10 μm. Each image is composed of 129 directly traced surface roughness profiles made over a 17.5 x 17.5 mm area of each sample.

a)



b)



a) Glass coverslip surface not treated (NT) on receipt from the supplier. The recording images for flame oxidised (FO), chloroform washed (CW) and cabbage-wax coated (CBW) glass coverslip surfaces were also indistinguishable from this image.

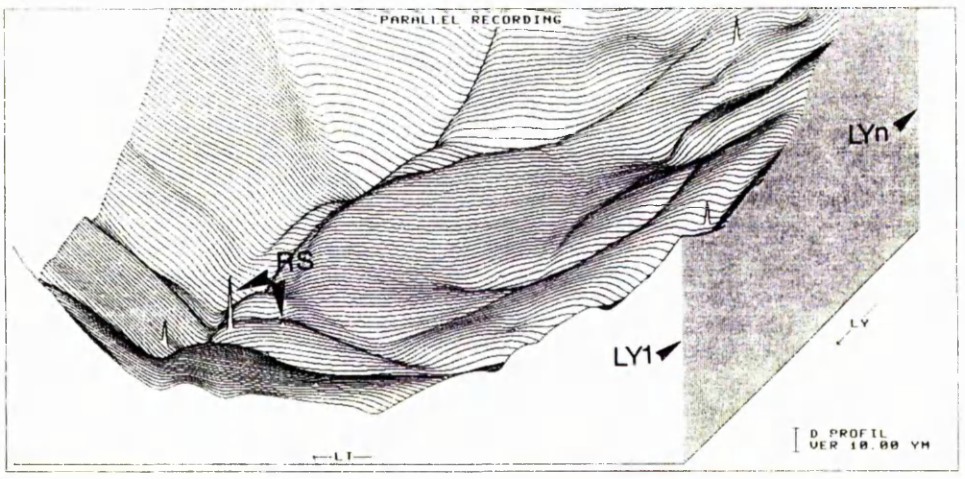
b) Acetate surface.

LY1 = first line trace, LYn = 129th line trace, Lt = direction of line trace.

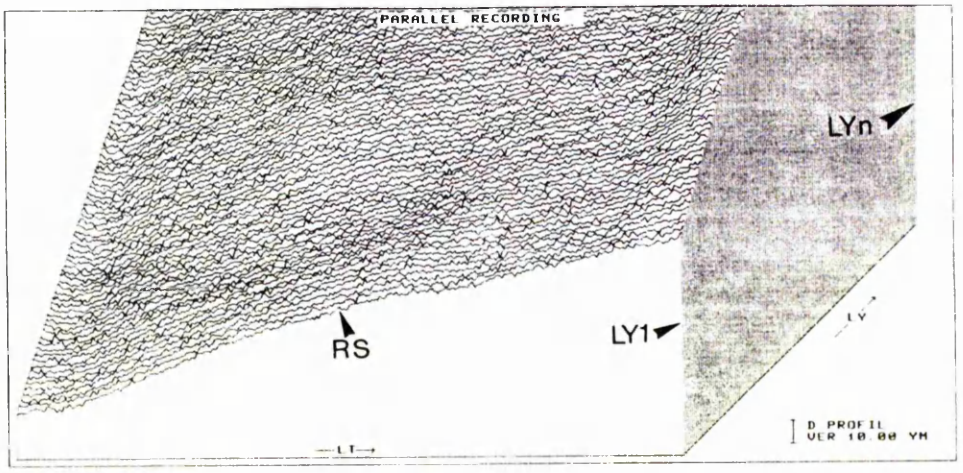
RS = sharp roughness spike. Bar shown is a 10 μm (YM) vertical depth scale.

Figure 3.10 continued.

c)



d)



c) Spurr resin surface

d) Dental wax surface. The recording image for plasma-glow discharged dental wax surfaces were indistinguishable from the image presented.

LY1 = first line trace, LYn = 129th line trace, Lt = direction of line trace.

RS = sharp roughness spike. Bar shown is a 10 μm (YM) vertical depth scale.

(Fig. 3.10.d) and PGD dental wax.

It should be noted that acetate, Spurr resin and dental wax samples, although appearing quite flat in appearance to the eye, each had an inherent large-scale irregularity (>25 μm) in their form (defined under ISO 1101 standard [Freeman & Richardson, 1990]). This irregularity was most apparent in the parallel recording image of the Spurr resin surface (Fig. 3.10.c). Here the centre of the sample was approximately 100 μm lower than its edges giving the recording image a sunken appearance. This was caused by the Spurr resin polymerising at different thicknesses on glass coverslip surfaces when formed (thicker at the edges of the coverslip than at the centre).

The parallel recording image for the acetate surface (Fig. 3.10.b) appeared undulating, this was also due to a large-scale irregularity caused by the sample being flexible and not laying perfectly rigid on the perthometer stage. This phenomenon may have also contributed to the large-scale irregularity seen in the dental wax surfaces, as these were produced on acetate sheets. Glass coverslip and CBW surfaces did not have any visible large scale irregularities, consequently the parallel recording image illustrated in Fig. 3.10.a appears very flat.

The large-scale form irregularities described above, are not a measure of surface roughness. Roughness is instead characterised by small-scale irregularities (<25 μm) of a surface (defined under DIN 4771 standard [Sander, 1991]). These irregularities appear in parallel recording images as sharp roughness spikes (SRS), either individually, as was the case for acetate (Fig. 3.10.b) and Spurr resin (Fig. 3.10.c) surfaces or in a continuous line, as is illustrated for dental wax (Fig. 3.10.d).

The distribution, size and relative frequency of small-scale roughness irregularities may be assessed using a combination of parallel recording images, and SEM micrographs (see Section 3.4.3 for SEM micrographs of each surface), and in addition, the surface roughness parameters Rz (mean peak to valley height), Rpm (mean levelling depth) and Ra (arithmetical mean roughness). Values of Rz, Rpm and Ra are presented for each surface in Table 3.11. (Note: see Figs. 2.3, 2.4 and 2.5 in Section 2.10.2 for derivation of surface roughness parameters Rz, Rpm and Ra respectively).

Table 3.11 Surface roughness parameter's Rz, Rpm and Ra, and the Rpm/Rz ratio measured for eight model surfaces.

Surface	Surface roughness parameter (μm)			
	Rz	Rpm	Ra	Rpm/Rz ratio
NT Glass coverslip	0.4 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	0.5
FO glass coverslip	0.4 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	0.5
CW glass coverslip	0.4 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	0.5
CBW glass coverslip	0.7 ± 0.1	0.5 ± 0.1	0.2 ± 0.0	0.7
Acetate	1.5 ± 0.2	1.2 ± 0.2	0.1 ± 0.0	0.8
Spurr resin	3.2 ± 0.5	1.5 ± 0.4	0.7 ± 0.1	0.47
Dental wax	18.7 ± 1.0	6.1 ± 0.7	2.0 ± 0.3	0.32
PGD Dental wax	17.9 ± 0.9	6.5 ± 0.8	1.9 ± 0.3	0.36

Rz = mean peak to valley height, Rpm = mean levelling depth, Ra = arithmetical mean roughness.

NT = not treated on receipt from supplier, FO = flame oxidised, CW = chloroform washed, CBW = cabbage-wax coated glass, PGD = plasma-glow discharged.

Data were recorded from 129 parallel traces made over the surface of one 17.5 x 17.5 mm sample of each model surface ± standard deviations.

Also presented in Table 3.11, is the Rpm/Rz ratio for each surface. Figure 3.11 illustrates how this ratio provides information on the profile shape of a surface. If the ratio is higher than 0.5, then the profile in question is sharp-ridged. If, however, the ratio is lower than 0.5, the profile is rounded and if the ratio is = 0.5, the profile is symmetrical about the mean line.

The roughness of each of the eight surfaces will now be described individually using surface roughness parameters, parallel recording and SEM images.

3.4.1.i Glass coverslip surfaces.

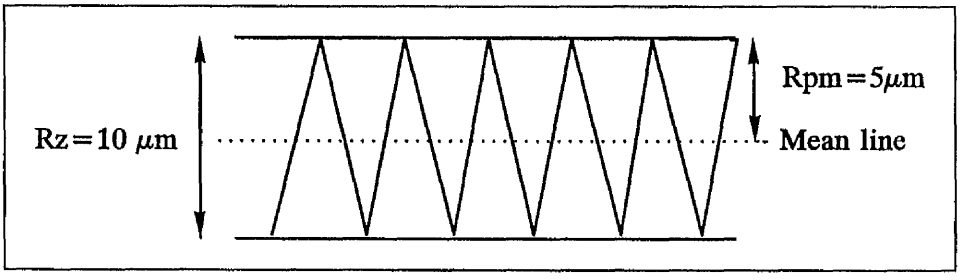
No distinction could be made between CW, FO and NT glass coverslip surfaces from either the parallel recording images (described previously, Fig. 3.10.a) or surface roughness values of Rz, Rpm or Ra (Table 3.11). For each of the three surfaces, Rz was very low at 0.4 μm. This indicates that any deviations of the profile were very small in magnitude and probably due to dirt or dust particles or even scratches on the glass surface. In addition, an undetectable Ra value and Rpm/Rz ratio of 0.5 indicates that these deviations were low in frequency and formed a symmetrical profile shape. These three surfaces may, therefore, be characterised as being very flat, with a very smooth bulk surface having a low frequency of small magnitude roughness areas. An SEM micrograph of a glass coverslip surface is illustrated in Plate 3.15 (Section 3.4.3.i).

3.4.1.ii Cabbage-wax coated glass coverslip (CBW) surfaces.

Although no distinction could be made between the parallel recording images of glass coverslip and CBW coated coverslip surfaces (Fig. 3.10.a); the Rz, Rpm and Ra values were all significantly higher than those for the un-coated glass surfaces described above. The Ra value increased from an undetectable level (un-coated glass surfaces) to 0.2 μm for the CBW surface. This indicates that the general roughness of the bulk surface had been increased. This increase was due to the presence of wax deposits, which were formed in different thicknesses over the glass surface. The difference in the thickness of the wax deposits introduced roughness into the surface; sharp peak and

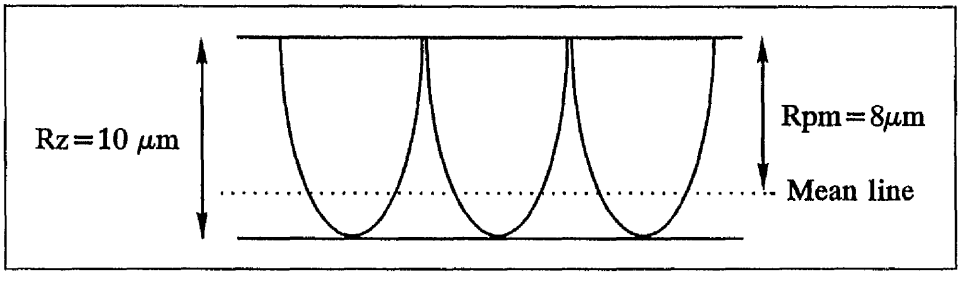
Figure 3.11 Diagrammatic representation illustrating how the Rpm/Rz ratio may be used to determine the profile shape of three different theoretical surface structures.

a) Symmetrical profile shape. Rpm/Rz ratio = 0.5.



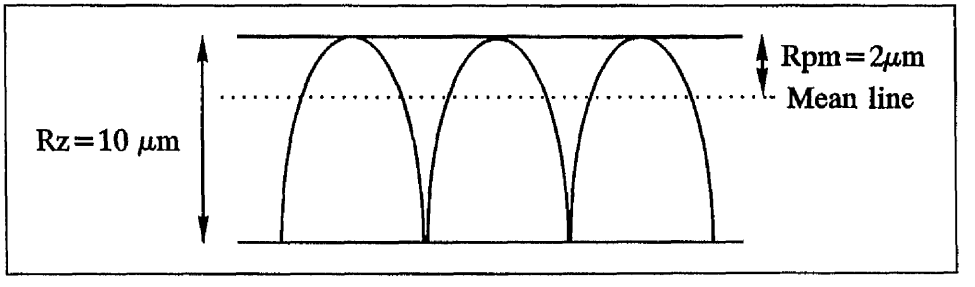
Rpm = 5 μm, Rz = 10 μm, Rpm/Rz ratio = 0.5.

b) Sharp ridged profile shape. Rpm/Rz ratio >0.5.



Rpm = 8 μm, Rz = 10 μm, Rpm/Rz ratio = 0.8

c) Rounded profile shape. Rpm/Rz < 0.5.



Rpm = 2 μm, Rz = 10 μm, Rpm/Rz ratio = 0.2

shallow valley areas being formed. These wax deposits are illustrated in the SEM micrograph illustrated in Plate 3.16.a-c (Section 3.4.3.i). This sharp peak profile shape is also supported by the high Rpm/Rz ratio of 0.7. The average distance between the sharp peak and shallow valley areas may be determined from the Rz value which was 0.7 μm.

3.4.1.iii Acetate surfaces.

The parallel recording image for the acetate surface (Fig. 3.10.b) indicated that the surface had a relatively high frequency of individual roughness spikes. From SEM micrographs (Plate 3.17.a & b, Section 3.4.3.i) it was shown that these roughness spikes were actually raised imperfections in the acetate surface. In SEM micrographs, the bulk of the acetate surface (Plate 3.17.b) did appear smoother than the CBW surface (Plate 3.16.b) and almost identical to glass (Plate 3.15). The average height of these imperfections is given by the Rz value of 1.5 μm. This is larger than the average peak to valley deviations of the CBW surfaces (0.7 μm). However, because the imperfections on the acetate were of a relatively low frequency, the Ra value of 0.1 μm for acetate was lower than the Ra value of 0.2 μm for the CBW surface. Obviously if the number of imperfections increased, then the Ra value would also increase and the acetate surface would become rougher than the CBW surface. These imperfections were also the source of the high Rpm/Rz ratio of 0.8 which indicates a sharp ridged profile shape.

3.4.1.iv Spurr resin surfaces.

The Rz and Ra values for the Spurr resin of 3.2 and 0.7 μm respectively, indicates that this surface was generally rougher than glass, CBW and acetate surfaces. It was, however, difficult to determine the source of this roughness from the parallel recording image (Fig. 3.10.c). It is possible that the high Rz value was the result of both the large scale form error, previously described for the surface, and the few large sharp roughness spikes observed in the parallel recording image. The source of these large spikes could not, however, be determined. It was possible that they represented dirt or hair particles that had contaminated and been incorporated into the surface during

polymerisation. The high Ra value can not, however, be explained by either large scale form errors or a low frequency of dirt particles. It is, therefore, likely that this relatively high Ra value originated from a high frequency of low volume deviations of the bulk surface. Such deviations were visible in SEM micrographs of the surface (Plate 3.18, Section 3.4.3.i). The bulk surface appeared rougher than glass (Plate 3.15) and acetate (Plate 3.17.a) surfaces and slightly rougher than CBW surfaces (Plate 3.16.a).

3.4.1.v Dental wax surfaces.

Untreated and PGD dental wax surfaces were indistinguishable from each other using either parallel recording images (Fig 3.10.d), surface roughness parameters (Table 3.11) or SEM micrographs (see Plate 3.19.a-c, Section 3.4.3.i for SEM micrographs of an untreated dental wax surface). SEM micrographs illustrate that the surfaces had a very complex rounded profile shape consisting of deep valley and rounded peak regions. This rounded profile shape is also indicated by the low Rpm/Rz ratio of 0.32. The average distance between these peak to valley areas may be determined from the very large Rz value of 18.7 μm. In addition, each peak to valley transect was also made up of smaller rounded peak to valley areas and the whole surface appears pitted at the $< 1\mu$m level (Plate 3.19.c). The combination of all of these surface roughness deviations is illustrated by the continuous line of sharp roughness peaks, visible in the parallel recording image (Fig 3.10.d) and the very high Ra value of 2.0 μm recorded for this surface. Dental wax surfaces were therefore rougher than the other seven model surfaces examined.

3.4.2 Assessment of surface hydrophobicity of leaf and model surfaces, by contact angle measurement.

Water contact angles for the eight model and two leaf surfaces described previously, were determined using Wilhelmy balance tensiometry (Section 2.10.3). Three replicate samples were analyzed for each surface type and, per sample, a three-cycle hysteresis force loop was recorded. A typical three-cycle force loop for each surface type is illustrated in Fig. 3.12.a-j. Within each hysteresis loop, three consecutive

Figure 3.12 Three-cycle Wilhelmy plate hysteresis force loop for each of eight model and two leaf surfaces.

Model surfaces.
a) Flame-oxidised glass, b) Not-treated glass, c) Chloroform-washed glass, d) Cabbage-wax coated glass, e) Spurr resin, f) Acetate, g) Dental wax, h) Plasma-glow discharged dental wax.

Leaf surfaces.
i) Lettuce, j) Cabbage.

For one replicate sample of each surface, one continuous (three-cycle) force loop is illustrated.

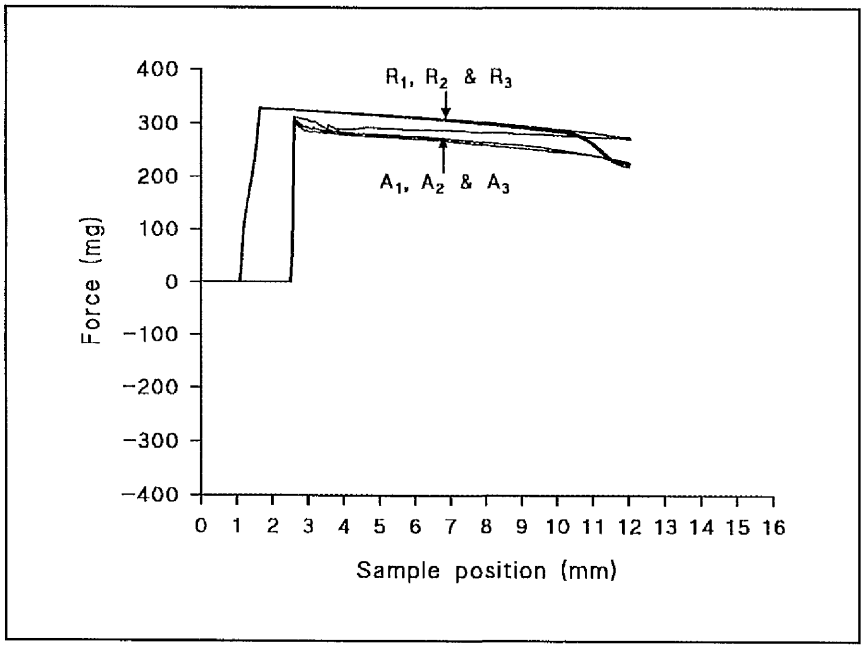
Each force loop consists of three advancing (A_{1-3}) and three receding (R_{1-3}) buoyancy slopes.

Water contact angles (Θ) calculated from each buoyancy slope at the Zero Depth Of Immersion (ZDOI) are presented.

Θa_{1-3} = Water contact angles derived from three consecutive advancing buoyancy slopes.
Θr_{1-3} = Water contact angles derived from three consecutive receding buoyancy slopes.

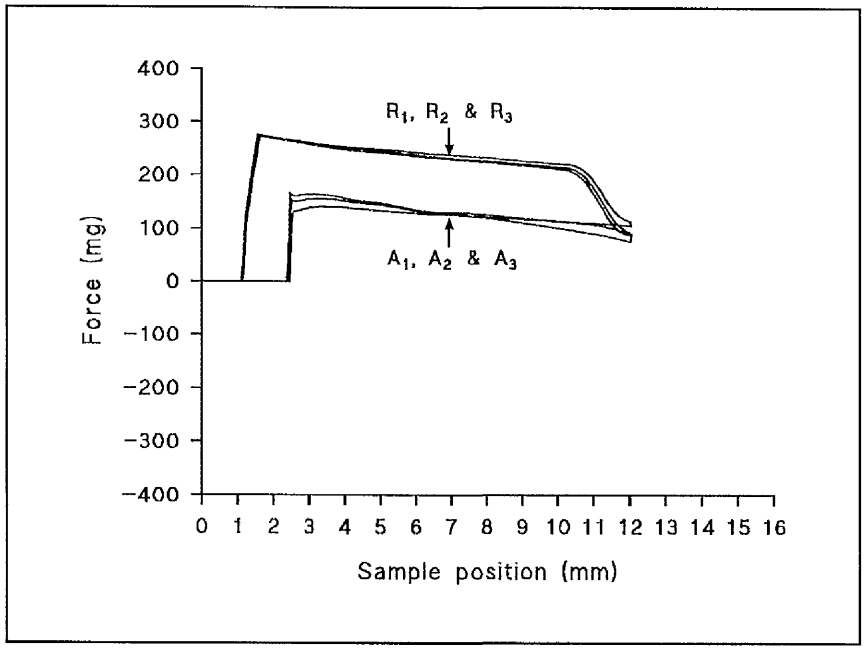
Figure 3.12.

a) Flame-oxidised glass coverslip (FO).



Θa_1=24.52°, Θa_2=26.32°, Θa_3=26.57°: Θr_1, Θr_2 & Θr_3 <0.00°.

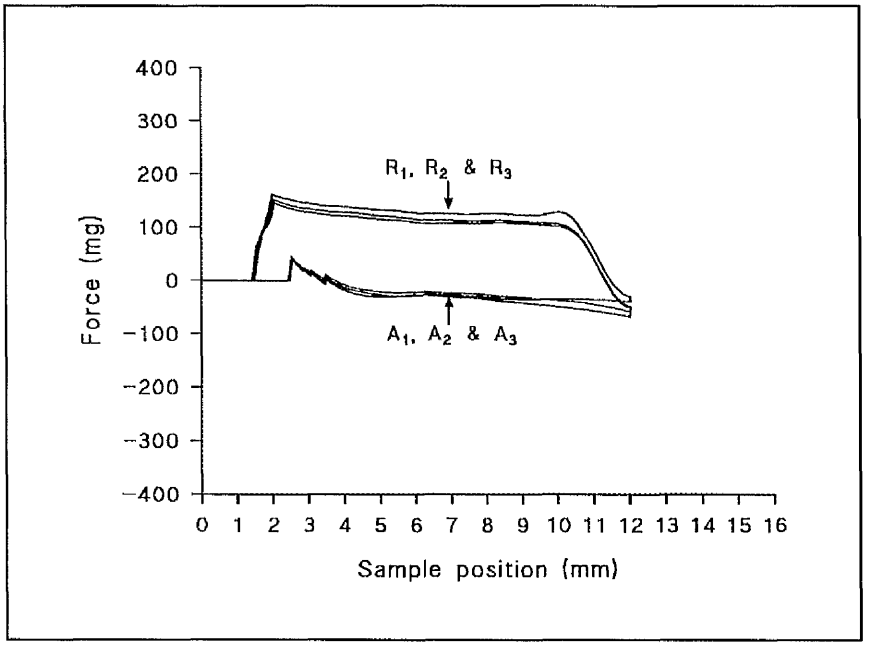
b) Not-treated glass coverslip (NT).



Θa_1=64.24°, Θa_2=59.71°, Θa_3=62.21°: Θr_1=37.13°, Θr_2=38.59°, Θr_3=38.54°.

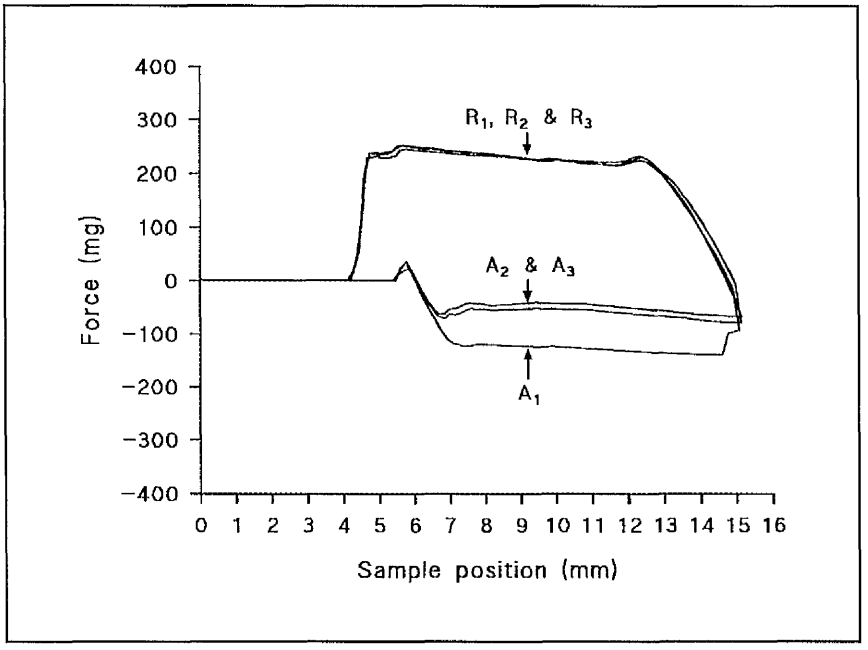
Figure 3.12. continued.

c) Chloroform-washed glass coverslip (CW).



Θa_1=93.58°, Θa_2=92.57°, Θa_3=92.02°: Θr_1=66.42°, Θr_2=68.12°, Θr_3=68.62°.

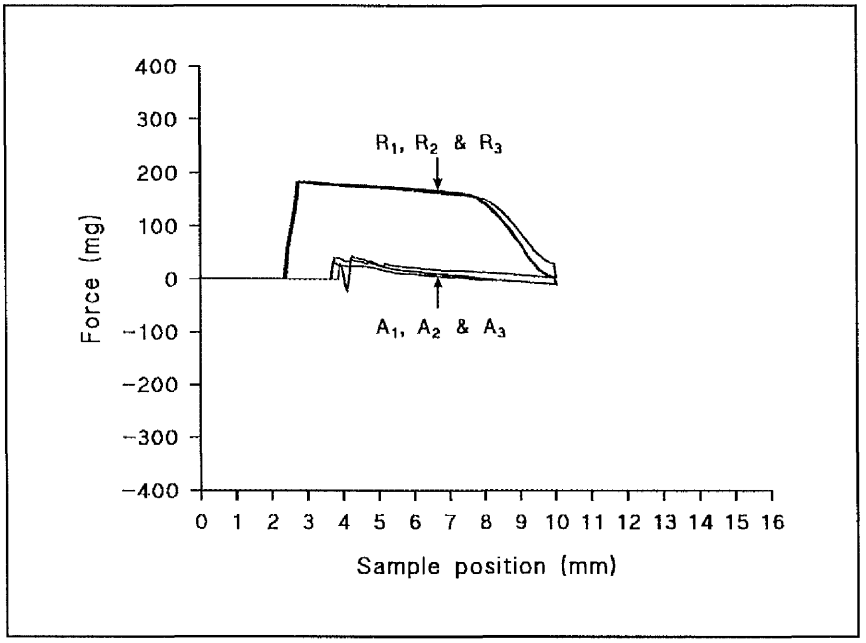
d) Cabbage-wax coated glass coverslip (CBW).



Θa_1=109.85°, Θa_2=97.86°, Θa_3=95.96°: Θr_1=42.36°, Θr_2=40.14°, Θr_3=39.84°.

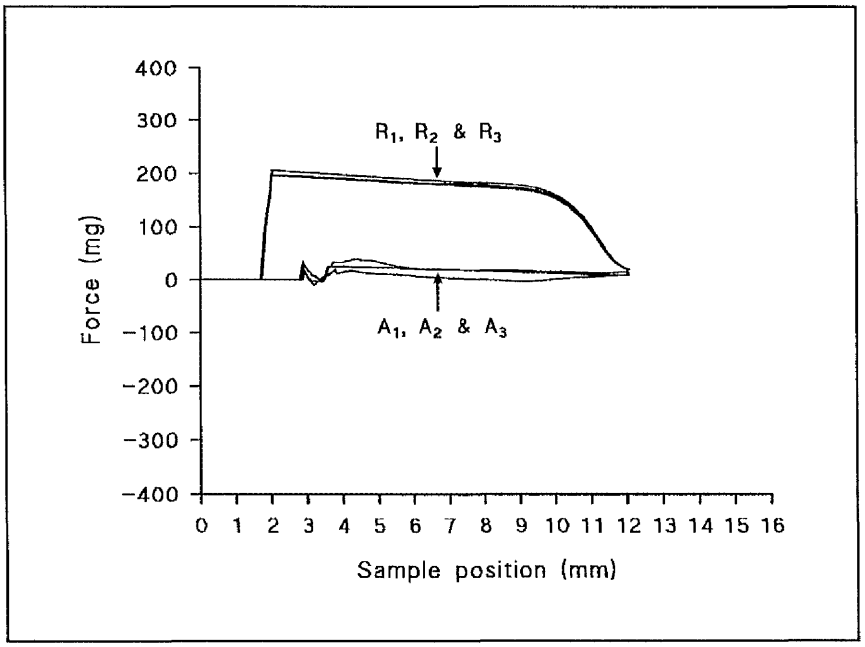
Figure 3.12. continued.

e) Spurr resin.



Θa_1=84.34°, Θa_2=86.61°, Θa_3=84.91°: Θr_1=56.87°, Θr_2=55.74°, Θr_3=55.15°.

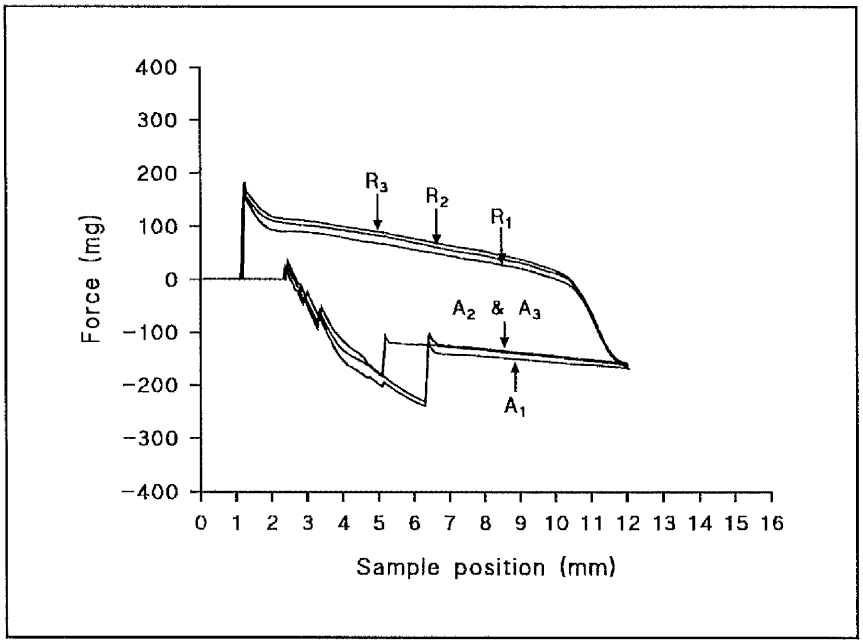
f) Acetate.



Θa_1=84.85°, Θa_2=85.58°, Θa_3=86.86°: Θr_1=53.48°, Θr_2=54.08°, Θr_3=52.11°.

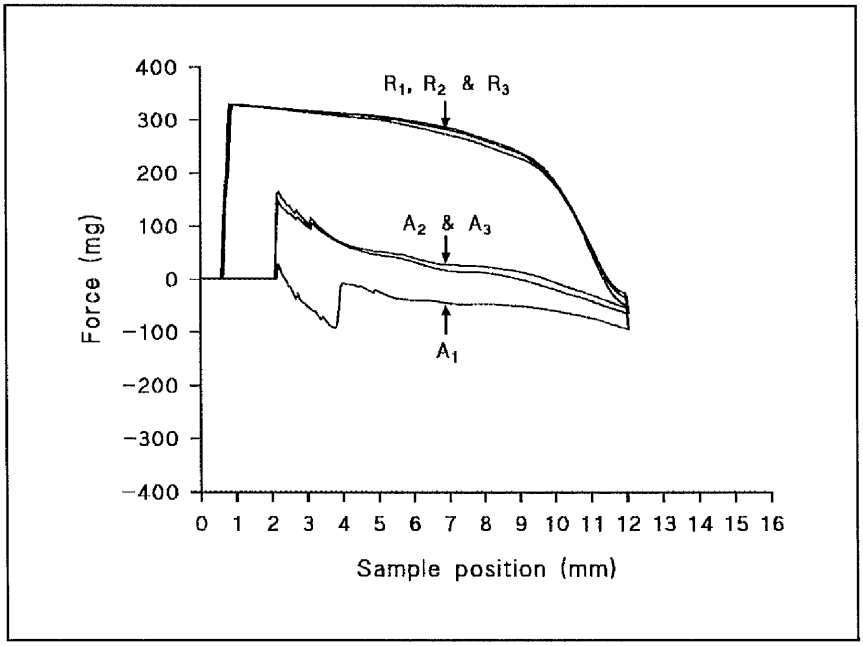
Figure 3.12. continued.

g) Dental wax.



$\Theta a_1 = 135.29°$, $\Theta a_2 = 130.80°$, $\Theta a_3 = 130.70°$: $\Theta r_1 = 71.21°$, $\Theta r_2 = 68.34°$, $\Theta r_3 = 65.25°$.

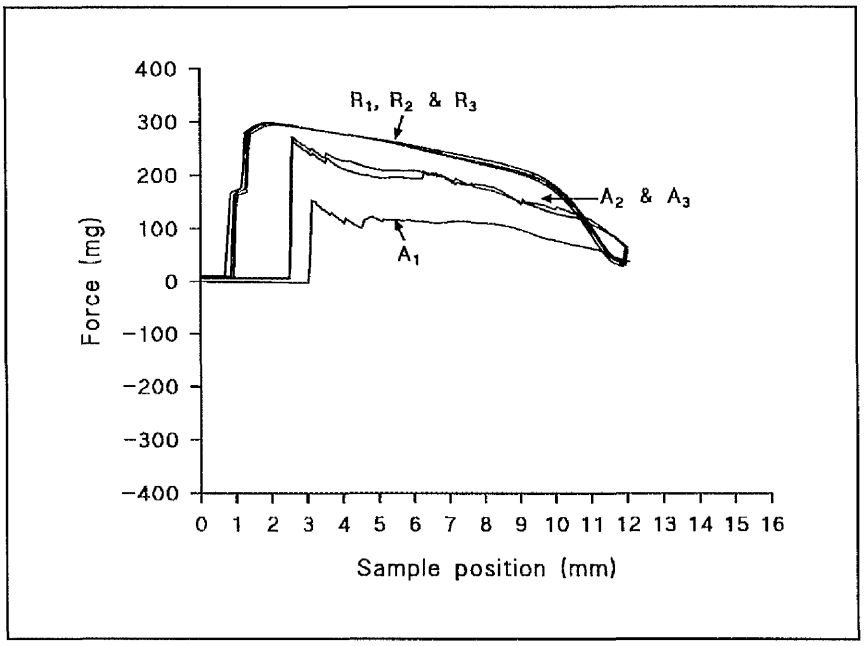
h) Plasma-glow discharged dental wax (PGD).



$\Theta a_1 = 93.62°$, $\Theta a_2 = 76.01°$, $\Theta a_3 = 75.91°$: Θr_1, Θr_2 & $\Theta r_3 < 0.00°$.

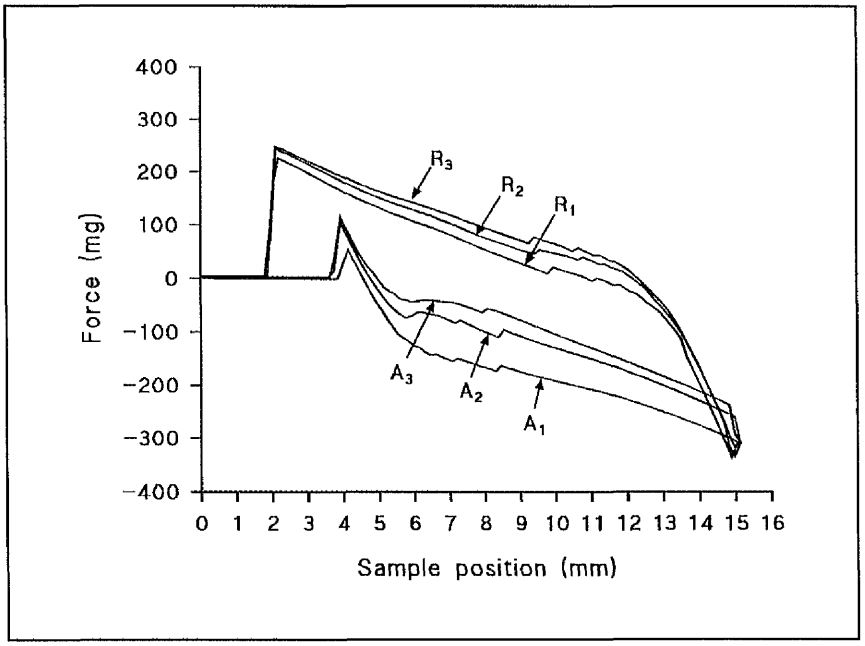
Figure. 3.12. continued.

i) Lettuce leaf tissue.



Θa_1=67.67°, Θa_2=43.44°, Θa_3=39.26°: Θr_1=25.33°, Θr_2=25.08°, Θr_3=24.24°.

j) Cabbage leaf tissue.



Θa_1=105.55°, Θa_2=91.81°, Θa_3=85.10°: Θr_1=61.67°, Θr_2=58.65°, Θr_3=55.67°.

advancing (A_{1-3}), and receding (R_{1-3}) buoyancy slopes are illustrated. The gradient of these slopes is dependent on the buoyancy of the sample, high gradients indicate high buoyancy.

As detailed in Section 2.10.3 (Fig. 2.7), wetting force at the Zero Depth of Immersion (ZDOI) were determined for each buoyancy slope and advancing (Θa) or receding (Θr) contact angles calculated. Consequently, three advancing (Θa_{1-3}) and three receding (Θr_{1-3}) contact angles were obtained for each replicate sample as are stated below Fig. 3.12.a-j. Wetting forces at the ZDOI of -318 mg, 0 mg and 318 mg, were equivalent to contact angles of 180°, 90° and 0° respectively.

As detailed in Section 3.4.2.i, hysteresis force loops Fig. 3.12.a-j, were used to categorised each surface on the basis of cycle-independent and cycle-dependent surface changes. Depending on this classification, mean advancing (MΘa) and receding (MΘr) contact angles for each surface type were calculated in either of two ways as also described in Section 3.4.2.i. Using these criteria, each surface is described separately in Section 3.4.2.ii.

3.4.2.i Characterisation of contact angle hysteresis.

All of the ten surface types exhibited contact angle hysteresis. Hysteresis was visible in all force loops (Fig. 3.12.a-j) as a vertical displacement between advancing (A) and receding (R) buoyancy slopes. Each surface type may be divided into either of two main group classifications, cycle-independent (thermodynamic) and cycle-dependent (kinetic) hysteresis, depending on the reproducibility of consecutive buoyancy slopes. [Note: A 3° difference between consecutive Θa or Θr contact angles is considered negligible and due to noise phenomena (Good, 1993)].

3.4.2.i.a Cycle-independent thermodynamic hysteresis.

Where both the advancing and receding buoyancy slopes were reproducible to 3° within both Θa_{1-3} and Θr_{1-3}, regardless of time or number of immersion cycles (i.e. $A_1 \equiv A_2 \equiv A_3$ and $R_1 \equiv R_2 \equiv R_3$), these surfaces are described as exhibiting only thermodynamic hysteresis. In Fig. 3.12.a, b, c, e & f, surfaces including FO glass, NT

glass, CW glass, Spurr resin and acetate respectively exhibited only thermodynamic hysteresis. This is indicated on each loop by the collective labelling of both advancing and receding buoyancy slopes (i.e. A_1, A_2 & A_3, and R_1, R_2 & R_3).

For these five surfaces, mean advancing (MΘa) and receding (MΘr) water contact angles were calculated as an average of all three immersion-cycles for three replicate samples (Table 3.12). This is justified because kinetic changes in these surfaces could be ignored.

Thermodynamic water contact angle hysteresis (▲Θ) for each surface type (Table 3.12) was calculated as the difference between mean advancing and receding contact angles (▲Θ = MΘa - MΘr).

3.4.2.i.b <u>Cycle-dependent kinetic hysteresis.</u>

The remaining five surfaces, namely, CBW glass, dental wax, PGD dental wax, lettuce and cabbage, all exhibited differences between first-cycle advancing (A_1) and receding (R_1) buoyancy slopes due to thermodynamic hysteresis and, in addition cycle-dependent changes ($>3°$), between consecutive advancing (A_1, A_2, A_3) and/or receding (R_1, R_2, R_3) buoyancy slopes due to kinetic hysteresis. The kinetic hysteresis for these five surfaces is indicated in Fig. 3.12. d, g, h, i & j respectively by independent labelling of buoyancy slopes whose inherent contact angles differ by more than 3°.

For these five surfaces, only the first advancing (Θa_1) and receding (Θr_1) contact angles for three replicate samples were used to calculate MΘa and MΘr (Table 3.12). Thermodynamic hysteresis ▲Θ was calculated as described above. Kinetic changes however, are illustrated by reporting second- (Θa_2, & Θr_2) and third- (Θa_3 & Θr_3) cycle contact angles, independently from first cycle contact angles (Fig. 3.12. d, g, h, i & j).

Table 3.12 **Mean advancing (MΘa), receding (MΘr) and water contact angle hysteresis (▲Θ) measured for model and leaf surfaces using Wilhelmy balance tensiometry.**

Substratum	Contact angle[3] MΘa[1]	MΘr[1]	▲Θ[2]
FO Glass	25.45 ± 1.14	≤ 0.00	25.45 ± 1.14
NT Glass	60.51 ± 4.37	38.59 ± 6.13	21.92 ± 2.65
CW Glass	92.72 ± 0.79	68.05 ± 1.60	24.67 ± 2.38
CBW coated coverslip	107.79 ± 2.11*	50.59 ± 8.10*	57.20 ± 10.21
Spurr resin	86.00 ± 1.11	57.37 ± 1.26	28.63 ± 1.21
Acetate	85.30 ± 0.41	50.63 ± 2.35	34.66 ± 1.94
Dental Wax	135.67 ± 3.47*	72.99 ± 2.12*	62.68 ± 2.33
PGD Dental Wax	100.91 ± 9.96*	≤ 0.00	100.91 ± 9.96
Lettuce	65.73 ± 1.21*	26.97 ± 2.00*	38.75 ± 2.29
Cabbage	98.32 ± 6.28*	55.16 ± 5.81*	43.16 ± 1.08

NT= Not treated on receipt from supplier, FO = Flame oxidised, CW = Chloroform washed, CBW = Cabbage-wax coated, PGD = Plasma-glow discharged.

[1]Mean advancing (MΘa) and receding (MΘr) water contact angles measured from three replicate samples of each surface type.

[2]Contact angle hysteresis (▲Θ) is the difference between MΘa and MΘr contact angles for the three replicate samples.

[3]Values for MΘa and MΘr contact angles are the means of three replicate immersion cycles for three replicate samples or, of only the first immersion cycle (*) for three replicate samples ± standard deviations.

3.4.2.ii Description of leaf and model surfaces in terms of water contact angles and contact angle hysteresis.

3.4.2.ii.a Surfaces exhibiting only thermodynamic hysteresis.

As described previously, five surfaces, namely, NT glass, FO glass, CW glass, Spurr resin and acetate exhibited a classical thermodynamic hysteresis as illustrated by reproducible advancing (A_{1-3}) and receding (R_{1-3}) buoyancy slopes in Fig. 3.12.a, b, c, e & f respectively. It should be recalled from Chapter 1, that there are two known sources of thermodynamic hysteresis, namely, roughness and chemical heterogeneity. Increases in either or both will result in an increase in the contact angle hysteresis.

Glass surfaces.

Flame oxidation treatment of glass will render the surface perfectly clean, homogenous and hydrophilic (Busscher, 1985). This was confirmed by the MΘr contact angle of $<0.00°$ measured for FO glass (Table 3.12). Chemical heterogeneity of this surface can therefore be ignored, and consequently, the MΘa contact angle of 25.45° and $\blacktriangle\Theta$ of 25.45°, can be defined as due to a very low surface roughness effect (as described by low Ra values in Section 3.4.1, Table 3.11). In addition, all surfaces in this study having a roughness greater or equal to that of FO glass, can be expected to have a $\blacktriangle\Theta \geq 25°$. The roughness of FO, NT and CW glass were all identical (Table 3.11); it is, therefore, not surprising that the $\blacktriangle\Theta$ for NT and CW glass were not significantly lower than approximately 25° (Table 3.12). In addition, since $\blacktriangle\Theta$ for NT and CW glass surfaces did not exceed 25°, it can be concluded that surface roughness was also their only source of hysteresis.

The MΘa contact angles for NT and CW glass of 60.51° and 92.72° respectively, were significantly higher than those of FO glass (Table 3.12). Similarly the MΘr contact angles for NT and CW glass of 38.59° and 68.05° respectively, were also significantly higher than FO glass (25.45°). Such increases in both MΘa and MΘr with constant hysteresis, indicates a progressive increase in the proportion of hydrophobic to hydrophilic groups for FO, NT and CW surfaces in turn. Increases in both MΘa and MΘr were possibly due to chemical masking of hydrophilic by hydrophobic groups; for

example, by forming a chemical-hydrophobic coating over a homogenous hydrophilic glass surface. This would increase the number of hydrophobic groups on the surface and decrease the number of hydrophilic groups exposed. Consequently increases in both MΘa and MΘr would occur but the hysteresis would remain stable due to surface roughness effects.

It can be concluded that FO glass was very hydrophilic, NT glass was moderately hydrophilic and CW glass was hydrophobic. Contact angle hysteresis was low for all three surfaces and due predominantly to roughness effects which were identical for all three surface types.

Spurr resin and acetate surfaces.

Spurr resin and acetate surfaces both had a MΘa contact angle of approximately 85° (Table 3.12), characterising them as relatively hydrophobic in the dry state. MΘr contact angles of 57.37° and 50.63° for Spurr resin and acetate respectively, illustrated that both surfaces became slightly hydrated following the first advancing immersion cycle. Hysteresis for Spurr resin of 28.63° was comparable to the 25° hysteresis for FO glass. Since Spurr resin was rougher than FO glass (Section 3.4.1), it probable that its hysteresis was also due to roughness effects and not chemical heterogeneity. Acetate surfaces were, however, smoother than Spurr resin but their hysteresis was higher at 34.66°. This unexpectedly high hysteresis may be explained by considering the effects of surface imperfections present on acetate surfaces (observed in SEM micrographs, Section 3.4.3.i, Plate 3.17.a & b). These imperfections may have increased ▲Θ via roughness and/or heterogeneity effects.

3.4.2.ii.b <u>Surfaces exhibiting both thermodynamic and kinetic hysteresis.</u>

Five surfaces, namely, CBW coated glass coverslips, dental wax, PGD dental wax, lettuce tissue and cabbage tissue exhibited both thermodynamic and kinetic hysteresis. Thermodynamic hysteresis, as already explained, was calculated for these surfaces as the difference between MΘa and MΘr contact angles each determined using only Θa_1 and Θr_1 contact angles. Kinetic hysteresis was characterised by cycle-

dependent changes within A_{1-3} and/or R_{1-3} buoyancy slopes, and will be described for one replicate of each surface type, using the comparison between Θa_1, Θa_2 and Θa_3, and/or, Θr_1, Θr_2 and Θr_3 contact angles presented below Fig. 3.12.d, g, h, i & j for each surface type respectively.

There are four known sources of kinetic hysteresis, namely, liquid penetration into the surface, reorientation of molecular groups at the solid/liquid interface, surface deformation, and surface mobility. All surfaces studied were however non-deformable, therefore this sources of kinetic hysteresis may be ignored.

Cabbage-wax coated glass coverslip surfaces (CBW).

CBW surfaces had high MΘa contact angles of 107.79° characterising them as hydrophobic in the dry state (Table 3.12). The MΘr contact angle of 50.59° for this surface type was, however, very low and consequently thermodynamic hysteresis was very high at 57.20° (Table 3.12). Since CBW surfaces were rougher than glass surfaces some of this hysteresis could be accounted for by roughness effects. However, CBW surfaces were smoother than Spurr resin (Table 3.11) but had a higher thermodynamic hysteresis (Table 3.12). This suggests quite a large chemical heterogeneity in CBW surfaces. It is, therefore, believed that CBW surfaces consisted of high numbers of both hydrophobic and hydrophilic sites leading to a high MΘa, low MΘr and consequent high $\blacktriangle\Theta$.

Kinetic hysteresis for CBW surfaces was characterised by cycle-dependent changes in advancing buoyancy slopes (Fig. 3.12.d). The majority of this change occurred between the first (A_1) and second (A_2) immersion cycles, resulting in a relatively small decrease of approximately 12° in Θa contact angle from Θa_1 to Θa_2 (Fig. 3.12.d). Θa contact angles remained constant at between 95° and 97° following the second (A_2) immersion cycle. This indicates that the surface was more hydrophobic before contact with water, but remained hydrophobic following initial wetting cycles.

Dental wax and PGD dental wax surfaces.

A MΘa contact angle for dental wax of 135.67° (Table 3.12) indicates that this

surface was very hydrophobic in the dry state. Plasma-glow discharge (PGD) treatment of dental wax decreased the MΘa contact angle to 100.91° indicating a decrease in the expression of hydrophobic groups on the surface. This decrease was not the result of a change in the roughness of the surface because, as determined in section 3.4.1, PGD treatment did not alter surface roughness measurements significantly. The MΘr contact angles for dental wax of 72.99° and consequent hysteresis of 62.68° (Table 3.12), was probably the result of high surface roughness effects. However, the MΘr contact angle of $\leq$ 0.00° for PGD dental wax and consequent very high hysteresis of $\geq$ 100° indicates that this surface was very hydrophilic in the wet state and very heterogenous with a high number of both hydrophobic and hydrophilic sites.

The kinetic change in the dental wax surface between consecutive immersion cycles was small, both advancing and receding contact angles decreased by approximately 5°, (Fig. 3.12.g). This decrease was most apparent between first and second immersion cycles and probably due to the adsorption or retention of water on the surface due to the rounded surface profile shape. For PGD dental wax, a large kinetic decrease of approximately 15-20° was observed between Θa_1 and Θa_2 contact angles (Fig. 3.12.h). This decrease was also probably the result of a retention of water on the surface. In this instance, however, the amount of water retained was higher compared to untreated dental wax surfaces due to the increased heterogeneity of the surface via the introduction of hydrophilic groups.

Lettuce and cabbage leaf surfaces.

MΘa contact angles of 65.73° and 98.32° for lettuce and cabbage leaf surfaces respectively, indicates that lettuce was hydrophilic and cabbage hydrophobic in the dry state. The relatively low MΘr contact angles of 26.97° for lettuce and 55.16° for cabbage tissue were probably due to the very complex surface morphology of leaf surfaces. It should be recalled that Wilhelmy balance tensiometry measured water contact angles as a mean value of both abaxial and adaxial leaf surfaces. Consequently, due to inherent variation between abaxial and adaxial surfaces, for example differences in the frequency and distribution of stomata, shape of epidermal cells etc. one would

expect quite a large contact angle hysteresis. The thermodynamic hysteresis for lettuce and cabbage tissue of 38.75° and 43.16° respectively, although being relatively large in comparison to glass (maximum of 25°), Spurr resin (28.63°) and acetate (34.66°) surfaces were, however, relatively small in comparison to CBW coated coverslip (57.20°) and PGD dental wax (100.91°). This suggests that in spite of morphological and structural differences between abaxial and adaxial leaf surfaces, chemical heterogeneity of each leaf surface was fairly limited.

The kinetic change in both lettuce and cabbage leaf surfaces was characterised by large decreases in consecutive advancing contact angles. A decrease of approximately 30° for lettuce and 20° for cabbage was observed between Θa_1 and Θa_3 contact angles (See Fig. 3.12.i & j for typical examples of hysteresis force loops of lettuce and cabbage leaf tissue respectively). In addition, cabbage leaf tissue also had changes of approximately 6° in receding water contact angles between Θr_1 and Θr_3. This decrease for receding contact angles was not observed for lettuce tissue, possibly due to the originally low Θr_1 contact angle of approximately 25°.

3.4.3 Localisation and quantification of adhering *L.monocytogenes* cells on model surfaces, determined using SEM analysis.

The adhesion of *L. monocytogenes* CRA 433 to glass coverslip [either flame oxidised (FO), Not treated (NT) or chloroform washed (CW)], cabbage-wax coated glass coverslip (CBW), Spurr resin, acetate and dental wax surfaces was assessed using the *in vitro* SEM adhesion assay detailed in Section 2.11. A comparison between the adhesion to dental wax and plasma-glow discharged dental wax is presented in a later section of this dissertation (Section 3.6.6).

One batch of *L.monocytogenes* CRA 433 cells was grown to early-stationary phase in TSB and resuspended in 1/4 strength Ringer's solution to a total cell concentration of 8.0×10^8 cells ml^{-1} as described previously for the inoculation of leaf surfaces (Sections 3.1.2 & 3.2). A 0.785 cm^2 area of each model surface was defined for inoculation (Section 2.11.1). Each area was inoculated (4.0×10^8 cells per area), incubated at 10°C for up to 24 h, washed with ten dips in distilled water to remove

planktonic cells and prepared for SEM examination (Section 2.11.2.i). Total adhering cells per cm^2 were quantified using SEM adhesion analysis as detailed in Section 2.11.2.ii.

SEM micrographs showing the localisation of adhering bacterial cells on each surface type following 24 h of incubation are presented in Section 3.4.3.i. Results for the quantification of adhering cells are presented in Section 3.4.3.ii.

3.4.3.i SEM analysis of inoculated model surfaces illustrating the localisation of adhering *L. monocytogenes* cells.

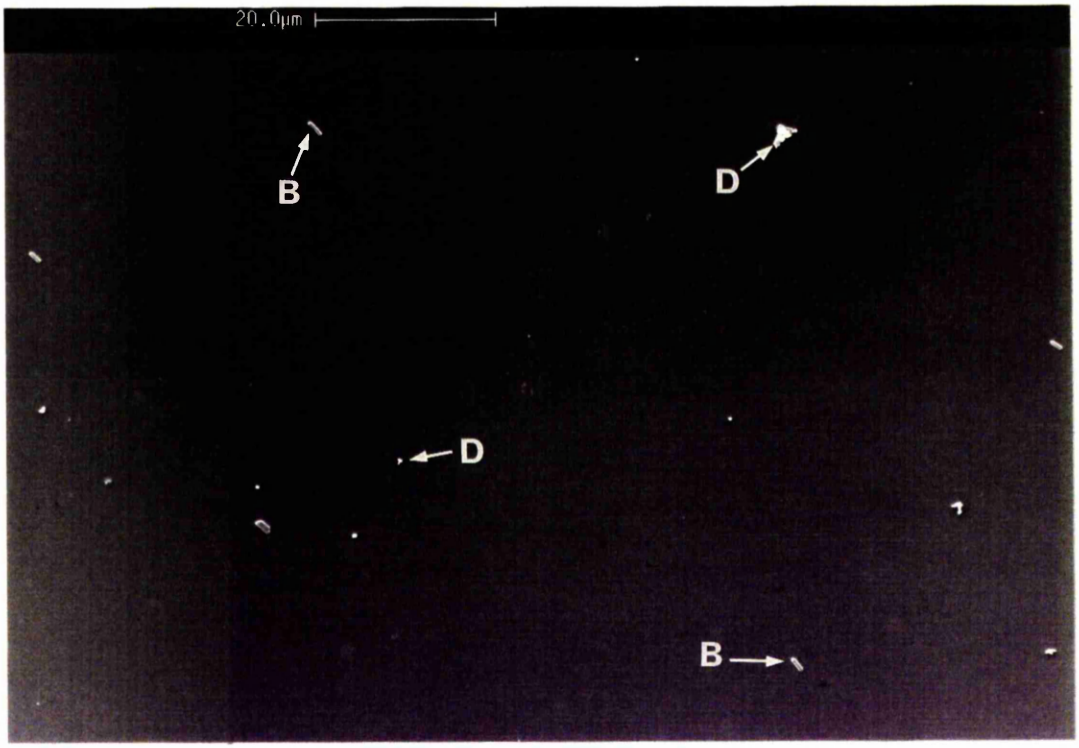
SEM micrographs of inoculated: chloroform-washed glass (Plate 3.15), cabbage-wax coated glass coverslip (CBW) (Plate 3.16.a-c), acetate (Plate 3.17.a & b), Spurr resin (Plate 3.18), and dental wax (Plate 3.19.a-c) show the distribution of adhering *L. monocytogenes* cells associated with each surface following a 24 h incubation period at 10°C. For each surface, the low magnification (x 1.0 K) micrograph illustrates an 8800 μm^2 area. This area is identical to that illustrated for inoculated cabbage (Plate 3.3.a) and lettuce leaf tissue (Plate 3.5.a). In addition, high magnification (x 2.00 K) micrographs of CBW coated glass (Plate 3.16.b), acetate (Plate 3.17.b) and dental wax (Plate 3.19.b) show identical areas of view to that shown for cabbage and lettuce leaf tissue illustrated in Plates 3.3.b and 3.5.b respectively.

3.4.3.i.a Glass surfaces.

Surfaces of FO and NT glass were identical in appearance to that illustrated for CW glass in Plate 3.15. As was predicted from the surface roughness measurement Ra of <0.00 (Section 3.4.1), the bulk of each surface was very smooth. Small dirt/dust fragments were visible on all glass surfaces (Plate 3.15). It is believed that these fragments largely contributed to the Rz roughness measurement of 0.4 μm (Table 3.11).

As will be described in Section 3.4.3.ii, of the three un-coated glass surfaces, CW glass, had the highest adhesion level ($= 4.1 \times 10^4$ cells cm^{-2}, at 24 h). Adhering cells usually occurred singly, and distributed randomly over the glass surface. Adhering cells did not show any specific localisation around dirt/dust fragments (Plate 3.15).

Plate 3.15 Scanning electron micrograph of a chloroform-washed glass coverslip surface (CW) inoculated with *L. monocytogenes* CRA 433 cells suspended in 1/4 strength Ringer's solution. The surface was incubated at 10°C for 24 h before being washed in distilled water.



A 8800 μm^2 area of an inoculated CW glass surface is shown. Dirt/dust fragments (D) are visible on the bulk glass surface.

A low number of bacterial cells (B) are distributed randomly over the glass surface.

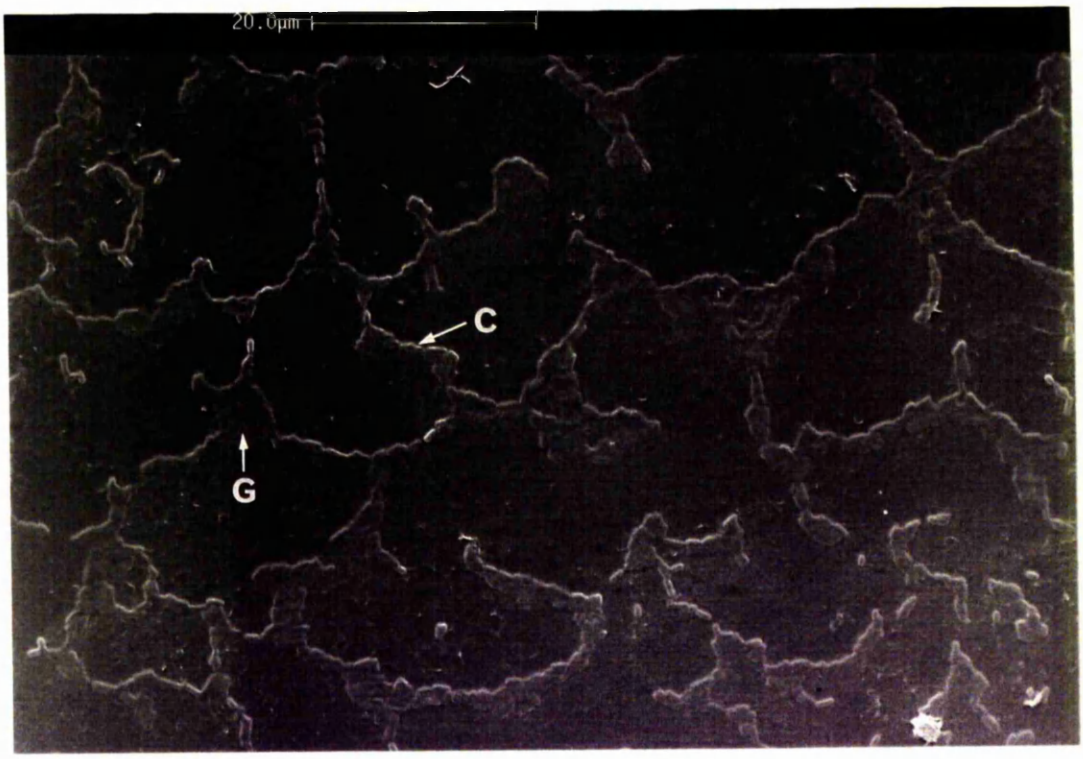
Plate 3.16 Scanning electron micrographs of a cabbage-wax coated glass coverslip surface (CBW) inoculated with *L. monocytogenes* CRA 433 cells suspended in 1/4 strength Ringer's solution. The surface was incubated at 10°C for 24 h before being washed in distilled water.

a) A 8800 μm^2 area of an inoculated CBW surface is shown.

b) A 2200 μm^2 area of an inoculated CBW surface is shown.

Bacteria adhered to the surface in groups (G) and chains (C) of cells to form a pattern of connecting rings on the CBW surface. Cabbage-wax deposits (D) are also indicated.

a)



b)

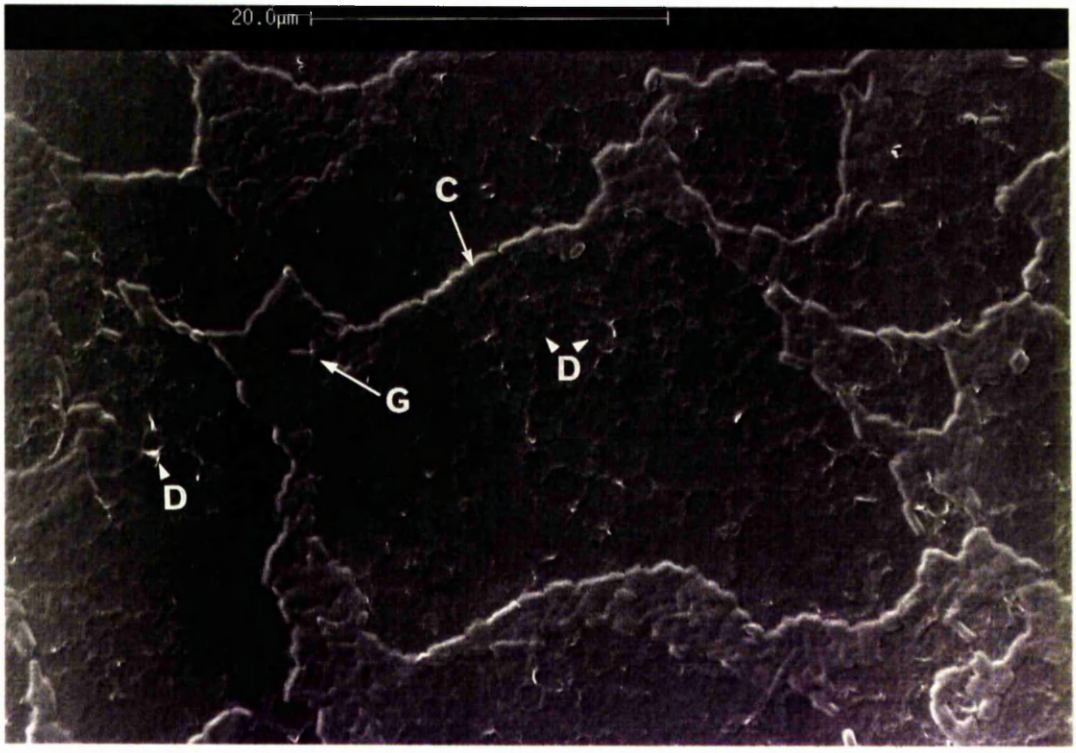
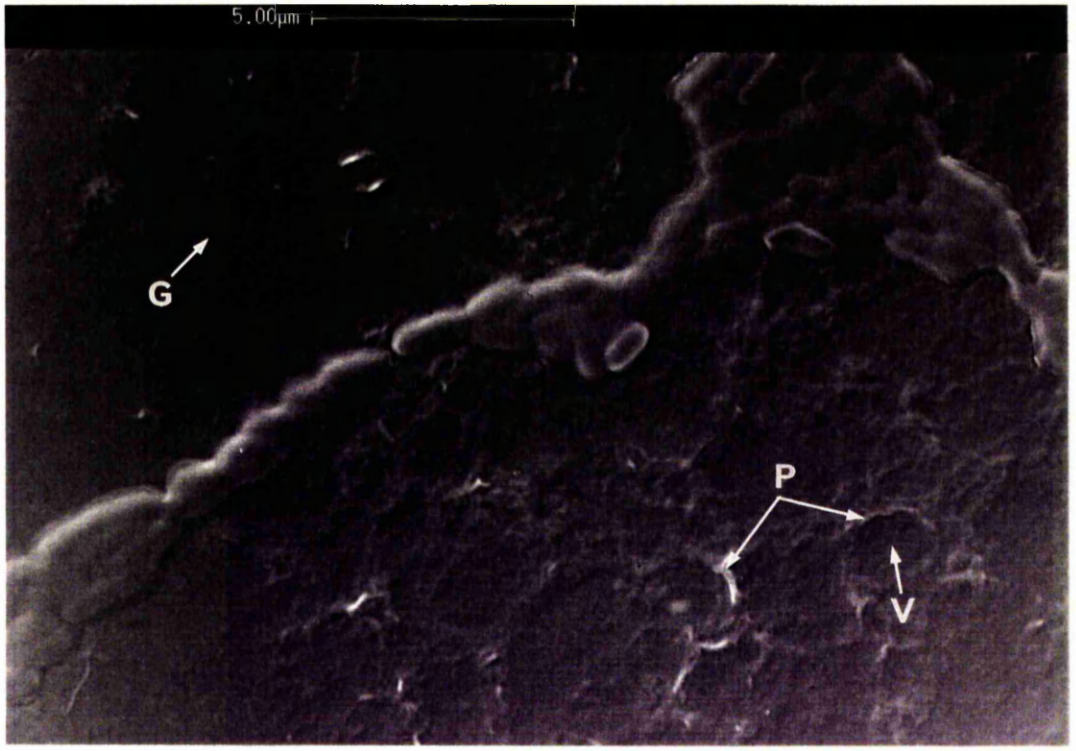


Plate 3.16 continued.



c) A 250 μm^2 area of an inoculated CBW surface is shown. Sharp-peak (P) and shallow-valley (V) areas of cabbage-wax deposits are illustrated which form a pattern of circular ridges on the coverslip surface. Wax deposits are very thin in certain areas (G), possibly exposing the underlying glass surface.

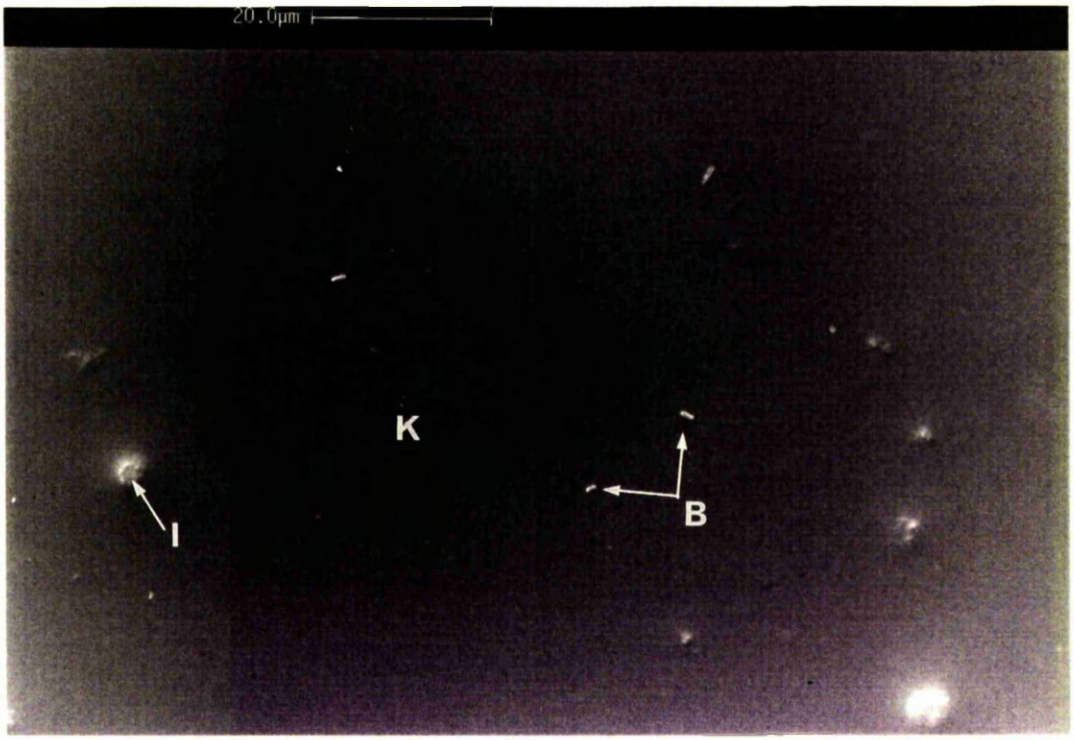
Plate 3.17 Scanning electron micrographs of an acetate surface inoculated with *L. monocytogenes* CRA 433 cells suspended in 1/4 strength Ringer's solution. The surface was incubated at 10°C for 24 h before being washed in distilled water.

a) A 8800 μm^2 area of an inoculated acetate surface is shown.

b) A 2200 μm^2 area of an inoculated acetate surface is shown.

The smooth bulk surface (K) and raised surface imperfections (I) are indicated.
A low number of adhering bacterial cells (B) are distributed randomly over the bulk acetate surface.

a)



b)

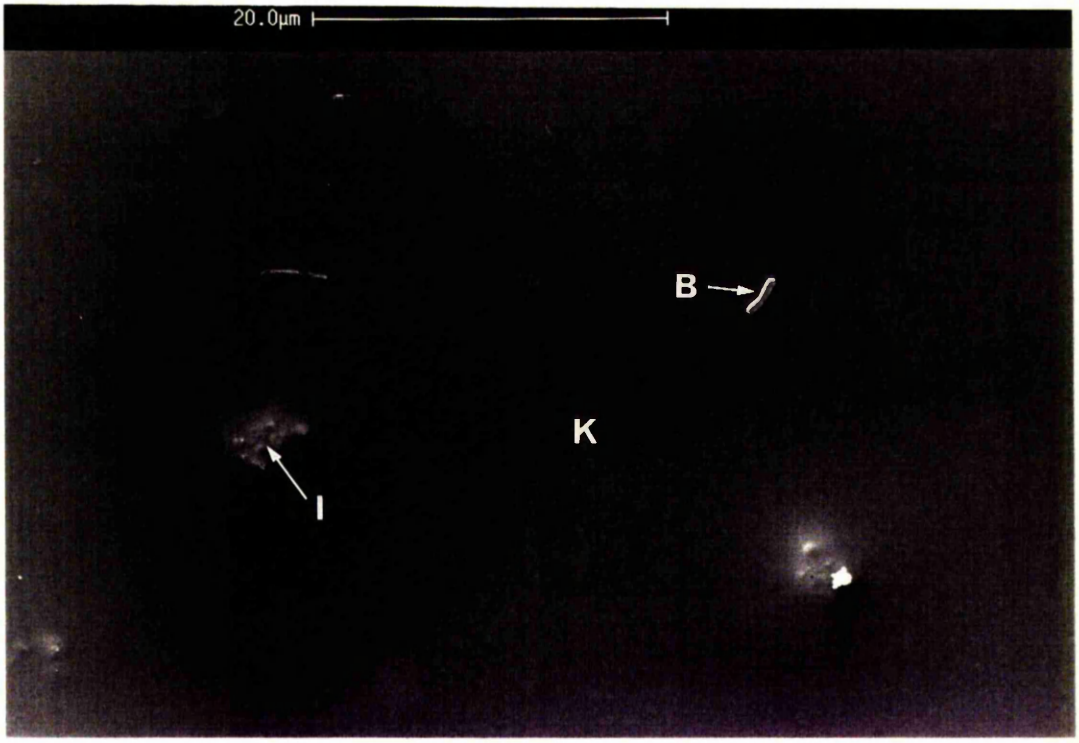
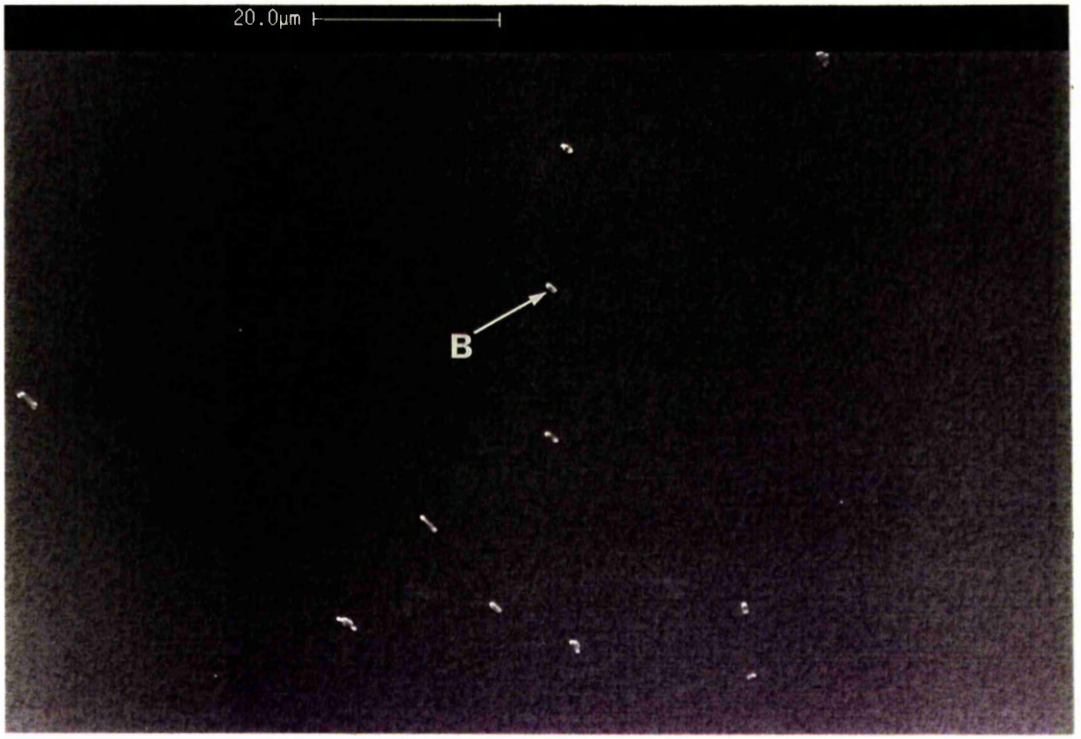


Plate 3.18 Scanning electron micrograph of a Spurr resin surface inoculated with *L. monocytogenes* CRA 433 cells suspended in 1/4 strength Ringer's solution. The surface was incubated at 10°C for 24 h before being washed in distilled water.



A 8800 μm^2 area of an inoculated Spurr resin surface is shown. Pocket (light) and peak (dark) regions can be seen forming a uniformly rough bulk surface. A low number of bacterial cells (B) are distributed randomly over the surface.

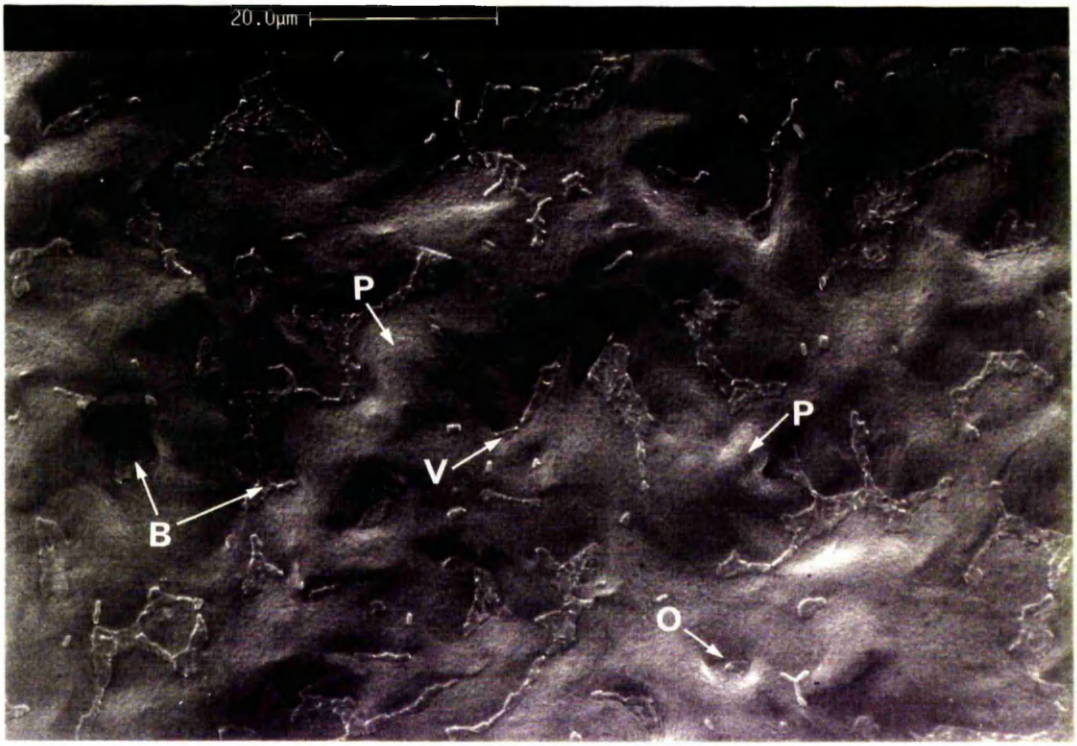
Plate 3.19 Scanning electron micrographs of a dental wax surface inoculated with *L. monocytogenes* CRA 433 cells suspended in 1/4 strength Ringer's solution. The surface was incubated at 10°C for 24 h before being washed in distilled water.

a) A 8800 μm^2 area of an inoculated dental wax surface is shown.

b) A 2200 μm^2 area of an inoculated dental wax surface is shown.

Deep-valley (V) and rounded-peak (P) regions of the dental wax surface formed a rounded profile shape. Each peak to valley transect consists of several smaller pockets (O). Adhering bacteria (B) are localised, mainly in groups, to valley and pocket regions.

a)



b)

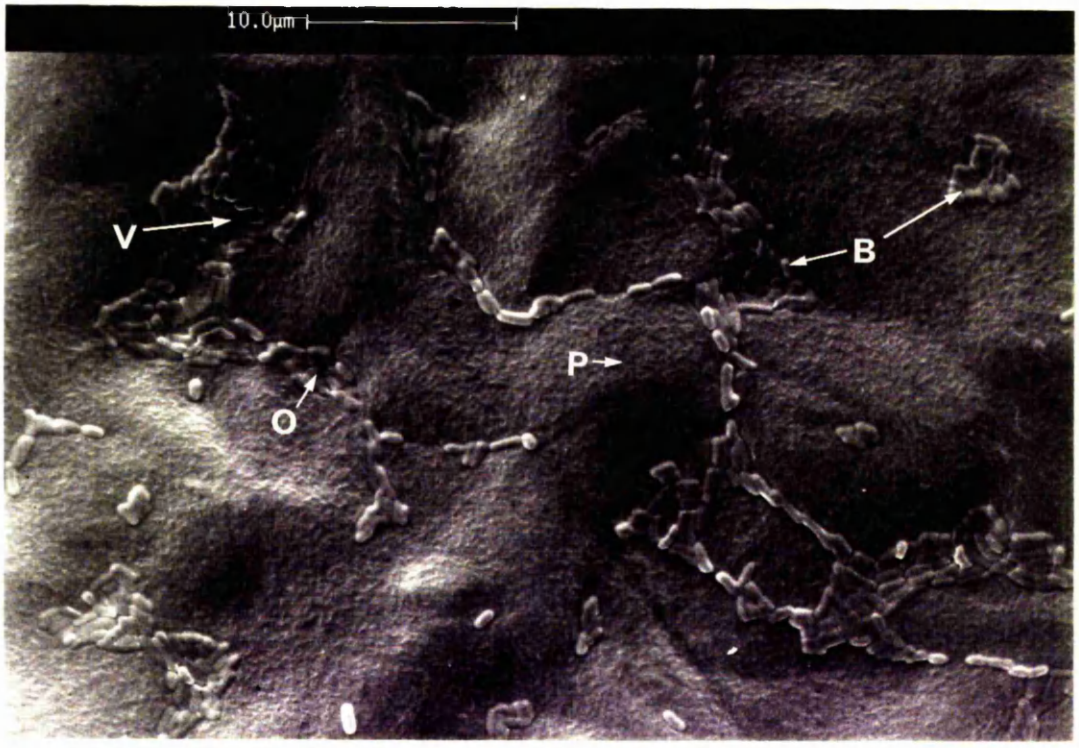
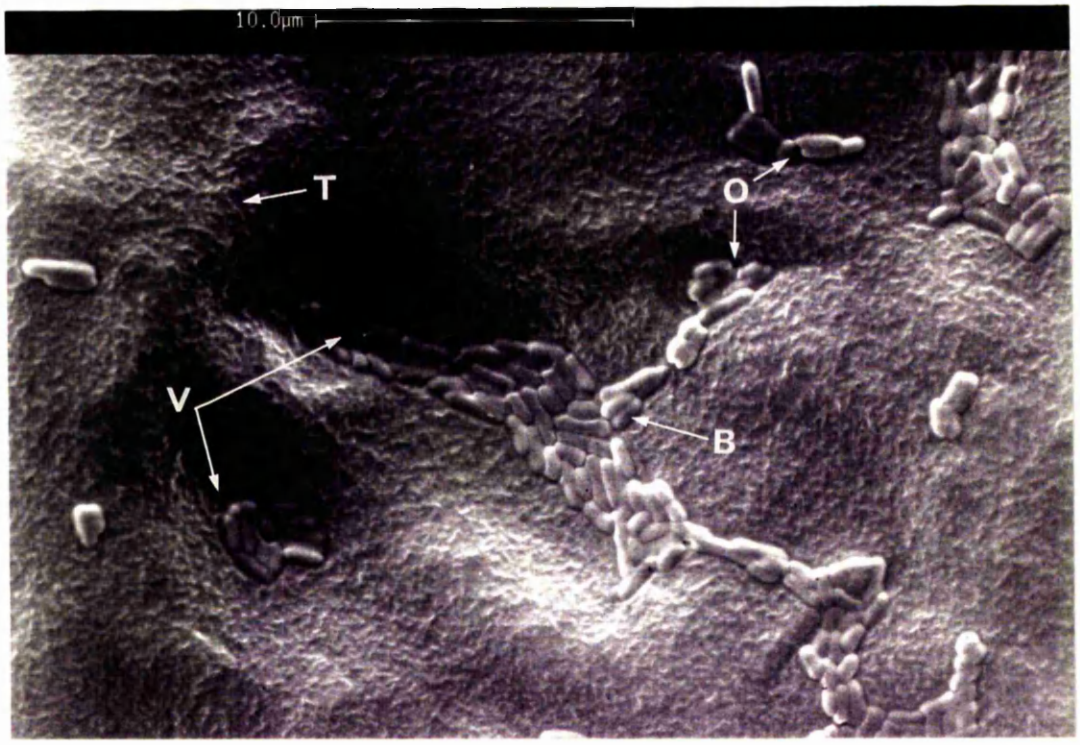


Plate 3.19 continued.



c) A 650 μm^2 area of an inoculated dental wax surface is shown. Groups of bacterial cells (B) are localised in pocket areas (O) of the surface. The whole dental wax surface is pitted (T).

3.4.3.i.b Cabbage-wax coated glass coverslip surfaces (CBW).

As detailed in Section 3.4.1 and as is illustrated in Plate 3.16.a-c, the roughness of CBW surfaces was dependent on the difference in thickness of the wax coating formed over the coverslip surface. Areas where the wax coating was relatively thick resulted in sharp-peak ridges. Similarly, where the coating was relatively thin shallow-valley areas were formed (Plate 3.16.c). The average distance in depth between peak and valley areas has previously been determined as 0.7 μm from Rz measurements (Section 3.4.1, Table 3.11). In addition, the combination of peak and valley areas formed an elaborate pattern of small (approximately 2 μm in diameter) circular ridges over the glass surface (Plate 3.16.c). Obviously CBW surfaces were rougher than un-coated surfaces. It should be noted that in some regions the wax deposits appeared very thin (Plate 3.16.c), possibly exposing the underlying glass surface.

Adhering *L. monocytogenes* cells occurred as a non-continuous mono-layer over the inoculated area (Plate 3.16.a). Adhering cells were distributed in an elaborate pattern of inter-connecting, almost circular rings (approximately 20-30 μm in diameter). These circular patterns of adjoining cells were composed of both small groups of cells (up to approximately 50 cells) arranged in either triangular or square blocks, and chains of individual or parallel cells lying end to end and radiating from the corners of adjacent cell blocks (Plate 3.16.b).

3.4.3.i.c Acetate surfaces.

Acetate surfaces had a very smooth bulk surface (Plate 3.17.a & b) which was comparable to un-coated glass (Plate 3.15) and smoother than CBW coated glass (Plate 3.16.a-c). Acetate surfaces did, however, also have quite a high frequency of imperfections in their bulk surface (Plate 3.17.b). As determined from Rz measurements (Section 3.4.1, Table 3.11), the average height of these imperfections was 1.5 μm.

Adhering cells occurred predominantly as single cells and were distributed randomly over the acetate surface. Adhering cells did not show any specific localisation around surface imperfections.

3.4.3.i.d Spurr resin surfaces.

Spurr resin surfaces appeared to have a uniform surface roughness which originated from a high frequency of pitted areas (Plate 3.18). The depth of these pits could not be determined from surface roughness analysis (Section 3.4.1) because of large-scale form irregularities in the surface. Spurr resin did, however, have a higher Ra value than all glass, CBW glass and acetate surfaces, indicating that its net roughness was higher than these five surfaces.

As for glass and acetate surfaces described above, adhering cells occurred singly and were randomly distributed over the Spurr resin surface (Plate 3.18).

3.4.3.i.e Dental wax surfaces.

Plate 3.19.a illustrates that dental wax had a rounded profile shape comprising deep-valley and rounded-peak regions, with an average vertical distance between each of 18.7 μm (determined from Rz measurements, Section 3.4.1, Table 3.11). As illustrated in Plate 3.19.a, the horizontal displacement between peak to peak areas was approximately 20-30 μm. Each peak to valley transect was composed of smaller pockets (Plate 3.19.b), each being approximately 5-10 μm in diameter. At high magnification (Plate 3.19.c) the whole wax surface appeared pitted. The depth of these pits could not be determined from SEM images or surface roughness analysis but each pit had a diameter of approximately 0.5 μm.

Adhering *L. monocytogenes* cells were localised to valley and pocket regions of the surface. Cells occurred singly in surface pockets but were more commonly observed in groups of adjacent cells lying along valley regions. A more detailed description of these mono-layers showing their formation in the presence of different electrolytes is detailed in Section 3.5.4.

3.4.3.ii Quantification of adhering *L. monocytogenes* cells on model surfaces, by SEM adhesion analysis.

Adhesion of *L. monocytogenes* to the seven model surfaces detailed previously was quantified using SEM adhesion analysis as detailed in Section 2.11.2.ii. For each

surface type, this involved counting the number of adhering bacteria present in ten randomly selected areas over the surface of each of three replicate samples. Adhering cells were quantified following 3 and 24 h of incubation and calculated as cells per cm^2 of surface.

3.4.3.ii.a Adhesion levels at 3 and 24 h for seven model surfaces.

Figure 3.13 illustrates adhesion levels to each of the seven model surfaces at 3 and 24 h following inoculation. Adhesion for all surfaces except FO glass, increased significantly from 3 to 24 h ($p < 0.05$, Student's t-tests). At both 3 and 24 h, adhesion to FO glass was below the minimum detection level of the assay (<1136 cells cm^{-2}). Between 3 and 24 h, adhesion increased by factors of 3.10 (NT glass), 4.66 (acetate), 4.22 (CW glass), 3.56 (Spurr resin), 69.21 (CBW coated glass) and 43.75 (dental wax).

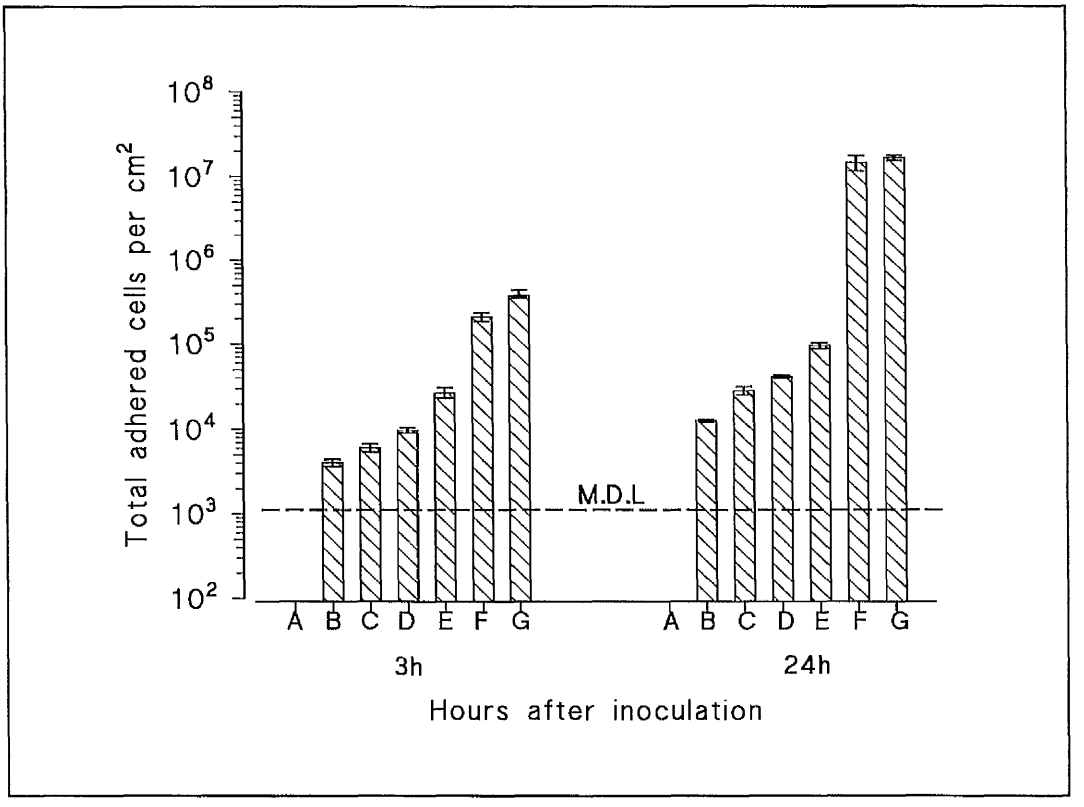
At both 3 and 24 h an identical trend of increasing adhesion from FO glass, NT glass, acetate, CW glass, Spurr resin, CBW coated glass to dental wax was observed. At 3 h, the difference in adhesion between these seven consecutive surface types was significant ($p < 0.05$, Multiple Range test). At 24 h the adhesion levels for CBW coated glass and dental wax were not significantly different ($p > 0.1$). Differences between the remaining five surface types were significant ($p < 0.05$) at 24 h. Adhesion to CBW coated glass and dental wax was approximately 160 times higher than adhesion to Spurr resin and 1250 times higher than that to NT glass.

SEM micrographs presented previously in Section 3.4.3.i, illustrate the increase in mean adhesion levels, at 24 h, from acetate (Plate 3.17.a) to CW glass (Plate 3.15) to Spurr resin (Plate 3.18) to CBW glass (Plate 3.16.a) and dental wax (Plate 3.19.a).

3.4.3.ii.b Correlation between adhesion levels at 24 h and the substratum surface roughness measurement Ra.

The roughness for each of the seven model surfaces was described in section 3.4.1 using Ra, Rz and Rpm measurements (Table 3.11). Of these three measurements, Ra values provide the best estimation of the mean roughness of each surface. In

Figure 3.13 Adhesion of *L. monocytogenes* CRA 433 cells suspended in 1/4 strength Ringer's solution to each of seven model surfaces following 3 and 24 h incubation at 10°C.



M.D.L = Minimum Detection Level (1136 cells cm^{-2})

Surfaces.
A = Flame oxidised glass (FO) (Numbers of adhering bacteria were below the M.D.L).
B = Not treated glass (NT).
C = Acetate.
D = Chloroform-washed glass (CW).
E = Spurr resin.
F = Cabbage-wax coated glass (CBW).
G = Dental wax.

Data represent the total number of adhering bacteria per cm^2 on each surface type 3 and 24 h after inoculation.

Values are the means of total bacterial counts made by SEM in ten random regions over the inoculated area of each of three replicate samples ± standard errors of the mean (bars).

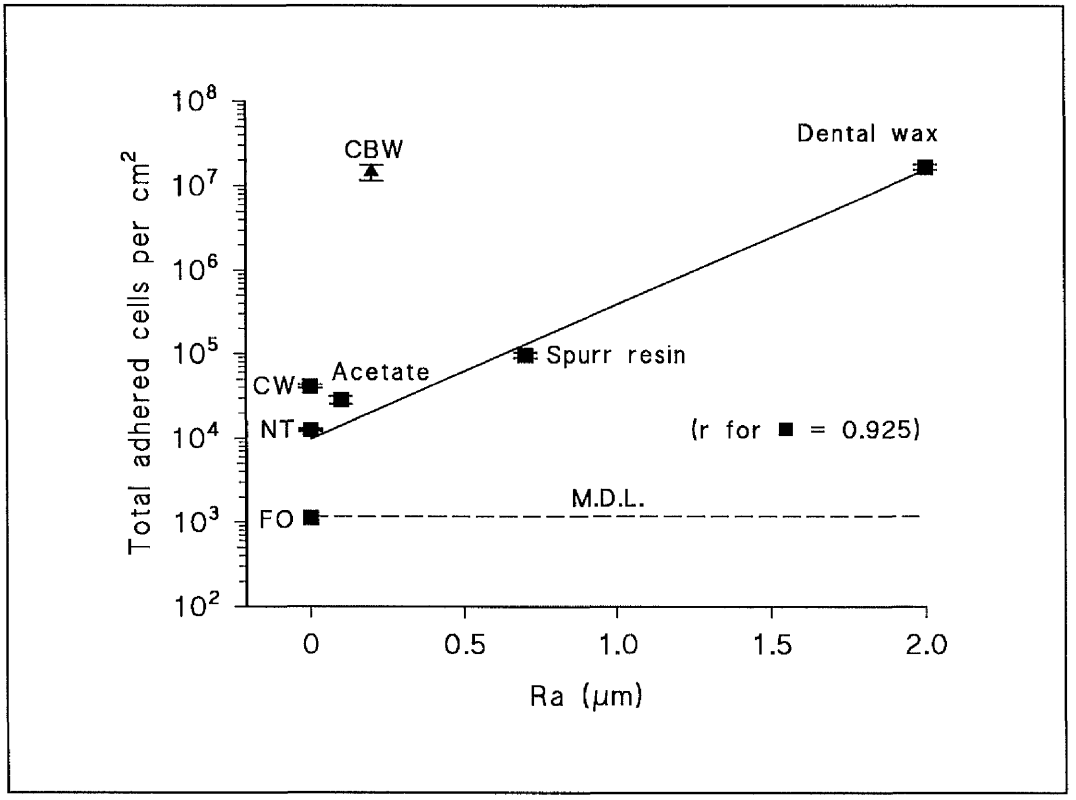
Fig. 3.14 the adhesion level for *L. monocytogenes* CRA 433 at 24 h, for each surface (determined in Section 3.4.3.ii.a) is plotted against the corresponding Ra roughness value.

In Fig. 3.14, a linear regression line with a correlation coefficient r = 0.925 is plotted using the data for six surfaces with the exclusion of the CBW coated glass. This regression line suggests a good positive correlation between adhesion and the Ra roughness measurement. However, when the data for CBW surfaces was included in the regression analysis with the other six surfaces, the linear correlation coefficient fell significantly from r = 0.925 to r = 0.660. As will become clearer in section 3.4.3.ii.c, a more complex situation involving the interplay of surface roughness and surface hydrophobicity appears to be controlling adhesion levels. The relatively high adhesion level to CBW with respect to the relatively low Ra value of this surface, may be the result of surface hydrophobicity influencing adhesion more strongly than surface roughness.

3.4.3.ii.c Correlation between adhesion levels at 24 h and substratum hydrophobicity.

The hydrophobicity of each of the seven model surface has previously been described using mean water contact angles (Section 3.4.2, Table 3.12). In Fig. 3.15, the adhesion level for *L. monocytogenes* CRA 433 at 24 h, for each surface type, is plotted against the mean advancing contact angle (MΘa) for that surface. A strong correlation between MΘa and adhesion levels was observed, the linear regression coefficient, r = 0.909. In general, high MΘa contact angles correlated with high adhesion levels. However, one exception to this observation was seen, namely that adhesion to Spurr resin (MΘa = 86.00°) was significantly higher ($p < 0.05$) than adhesion to CW glass (MΘa = 92.72°). This may be accounted for by considering the relatively high surface roughness of Spurr resin (Ra = 0.7) compared to CW glass (Ra = 0.0) (Section 3.4.1). In this instance, the increase in surface roughness between these two surfaces probably contributed more strongly to adhesion levels than did the increase in surface hydrophobicity.

Figure 3.14 Correlation between the adhesion of *L. monocytogenes* CRA 433 to seven model surfaces at 24 h and the roughness of each surface as described by Ra measurements.



Surfaces.
Flame oxidised glass (FO), Not treated glass (NT), Chloroform-washed glass (CW), Acetate, Spurr resin, Cabbage-wax coated glass (CBW), Dental wax.

———— Linear regression correlation coefficient, r = 0.925 (calculated for ■ data set only).

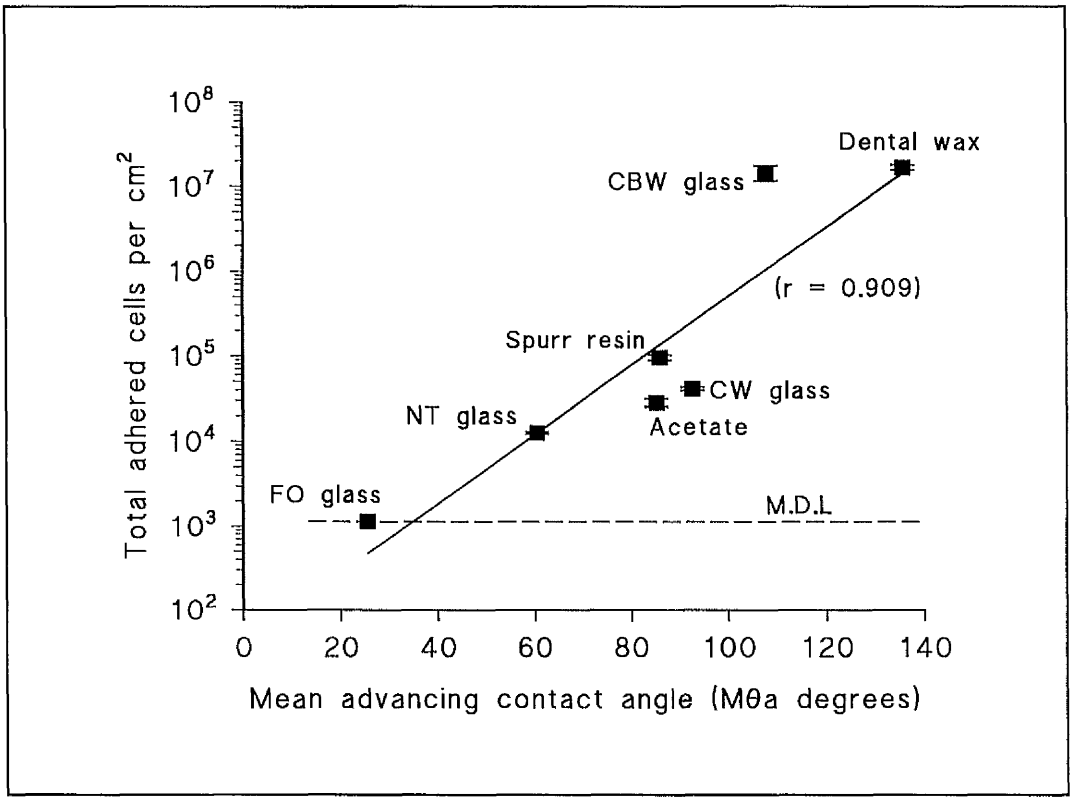
M.D.L. = Minimum Detection Level (1136 cells cm^{-2}).

Note: The adhesion to FO glass was below the M.D.L but is plotted at the M.D.L.

Values of Ra for each surface type were obtained in Section 3.4.1 and are summarised in Table 3.11.

Values for adhesion are the means of bacterial counts made by SEM in 10 random areas over the surface of three replicate samples. Bars shown are standard errors of the mean.

Figure 3.15 Correlation between the adhesion of *L. monocytogenes* CRA 433 to seven model surfaces at 24 h and the hydrophobicity of each surface as measured from mean advancing water contact angles.



Surfaces.
Flame oxidised glass (FO), Not treated glass (NT), Chloroform-washed glass (CW), Acetate, Spurr resin, Cabbage-wax coated glass (CBW), Dental wax.

———— Linear regression correlation coefficient, r = 0.909.

M.D.L. = Minimum Detection Level (1136 cells cm^{-2}).

Note: The adhesion to FO glass was below the M.D.L but is plotted at the M.D.L.

Data represent the mean adhesion level for seven surfaces at 24 h, plotted against the mean advancing water contact angle (MΘa) of each surface (MΘa as determined in Section 3.4.2 and summarised in Table 3.12).

Values for adhesion are the means of bacterial counts made by SEM in ten random areas over the surface of three replicate samples. Bars shown are standard errors of the mean.

3.4.3.ii.d Comparison between percentage adhesion levels to model and leaf surfaces.

For comparison with previous adhesion experiments on leaf tissue, adhesion data at 24 h for model surfaces presented in Section 3.4.3.ii.a was also expressed as a percentage adhesion level of the total cells ml^{-1} inoculated onto each surface at 0 d (i.e. no. adhering cells $cm^{-2}/8.0 \times 10^8$ cells ml^{-1}). These percentage adhesion values are presented in Table 3.13.

Percentage adhesion levels obtained from three experiments on lettuce tissue have previously been summarised in Table 3.6 (Section 3.2.4.ii). At 1 d following inoculation, percentage adhesion levels for cells suspended in 1/4 strength Ringer's solution ranged from 0.267% to 0.333% (S.E.= 0.041 to 0.213). Similarly, a percentage adhesion level of 4.513% (S.E.= 0.544) was calculated for cabbage leaf tissue at 1 d (Section 3.2.4.ii, Table 3.5). It should be recalled that these adhesion values were calculated from viable adhering cells, whereas those for model surfaces (Table 3.13) were calculated from total adhering cells. However, it has been shown for cabbage (Section 3.4.2.i.a) that the total adhering cell population was not significantly different to the viable adhered population. Although this could not be confirmed for lettuce tissue, due to problems of quantifying total adhering cells (Section 3.1.2.i.b), the same situation is possible.

A comparison between percentage adhesion levels for model and leaf surfaces is, therefore, possible for cells suspended in 1/4 strength Ringer's solution. From Table 3.13, it is apparent that CBW coated glass and dental wax have percentage adhesion levels which were not significantly different to each other, but higher by a factor of approximately 6-7 than lettuce, and approximately half that of cabbage tissue. Adhesion levels for all other model surfaces were more than 17.5 times lower than that of lettuce.

Table 3.13 Percentage adhesion levels *L. monocytogenes* CRA 433 cells, suspended in 1/4 strength Ringer's solution, to model surfaces 24 h after inoculation.

Substratum	% Adhesion
Flame oxidised glass (FO)	< 0.00014
Not treated glass (NT)	0.00157 ± 0.0001
Acetate	0.00353 ± 0.0004
Chloroform washed glass (CW)	0.00522 ± 0.0002
Spurr resin	0.01884 ± 0.0009
Cabbage wax coated glass (CBW)	1.814 ± 0.325[a]
Dental wax	2.074 ± 0.144[a]

Data represent the numbers of total adhering cells detected (per cm^2) 24 h following inoculation expressed as a percentage of the total cells (per ml) inoculated on to the surface at 0 h.

Values are the average of total bacterial counts made in ten random areas on each of three replicate samples ± standard errors of the mean.

Values followed by the same letter are not significantly different from each other ($p < 0.05$, Multiple Range test).

3.5 Adhesion of *L. monocytogenes* to dental wax surfaces; quantification using SEM adhesion analysis.

All further adhesion experiments examined the adhesion of *L. monocytogenes* to dental wax surfaces only. Unless otherwise stated, inoculum for all experiments was prepared by incubating *L. monocytogenes* cells in TSB at 10°C until early-stationary phase. Cells were washed x3 with distilled water and resuspended to a total cell concentration of 8.0×10^8 cells ml^{-1} in the required diluent (detailed separately for each experiment). All surfaces were incubated at 10°C and unless otherwise stated, following incubation were subject to the standard washing procedure of ten dips in distilled water to remove non-adhering cells.

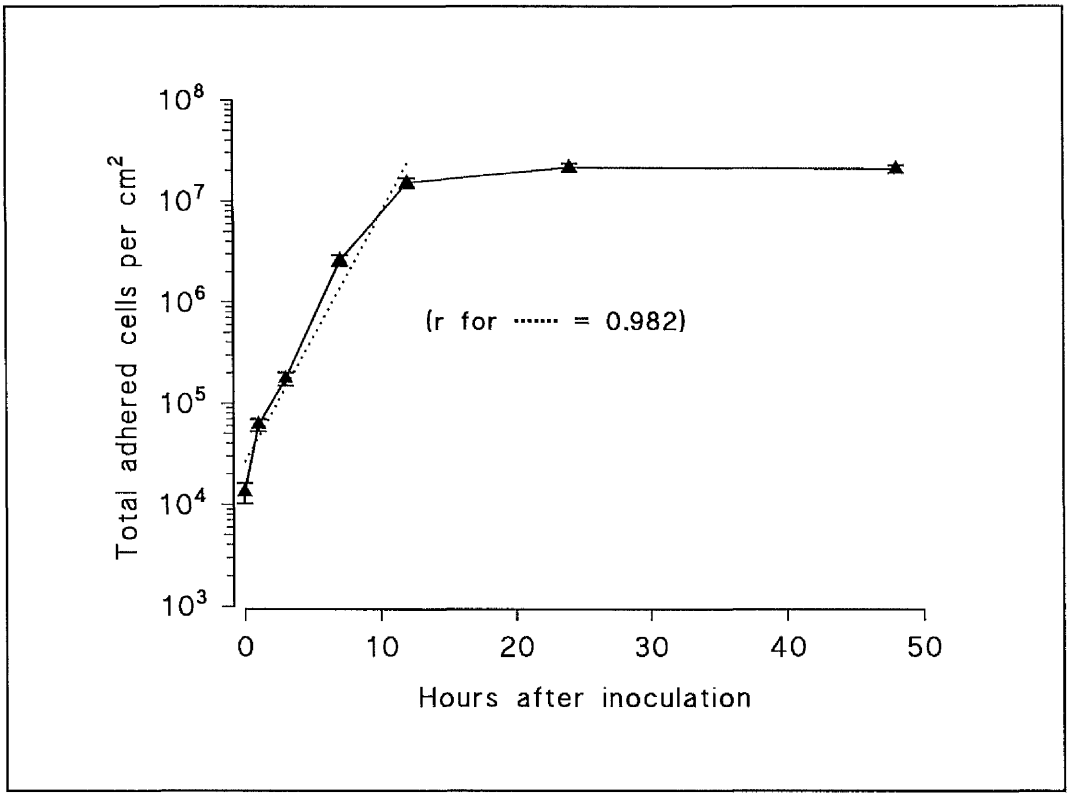
For all experiments described in Sections 3.5.1 to 3.5.6.ii, adhesion to dental wax was assessed using the SEM adhesion analysis protocol (detailed in Section 2.11). This protocol has been used in section 3.4.3 for the analysis of adhesion to different model surfaces. From section 3.6 onwards, the *in vitro* photometric adhesion assay (detailed in Section 2.12) was developed, and used to rapidly quantify adhering *L. monocytogenes* cells on dental wax by crystal violet staining.

3.5.1 Adhesion of *L. monocytogenes* to dental wax surfaces over a 48 h incubation period at 10°C.

Dental wax surfaces were prepared, and inoculated with *L. monocytogenes* CRA 433 cells suspended in 1/4 strength Ringer's solution. Surfaces were incubated for 0, 1, 3, 7, 12, 24 and 48 h when triplicate surfaces were washed and prepared for SEM adhesion analysis.

Adhesion levels over the 48 h incubation period are illustrated in Fig. 3.16. Immediately following inoculation (0 h), a relatively small number (1.3×10^4 cells cm^{-2}) of *L. monocytogenes* cells immediately adhered to the wax surface. Over the first 12 h of incubation, adhered cells increased at a logarithmic rate. This increase was closely correlated to the linear regression line (dotted line in Fig. 3.16) with a linear correlation coefficient, $r = 0.982$. Between 12 and 24 h of incubation, the increase in adhering cells became linear, this increase was significant for $p < 0.1$ (Student's t-test) but not

Figure 3.16 Adhesion of *L. monocytogenes* CRA 433 cells suspended in 1/4 strength Ringer's solution to dental wax over 48 h incubation period at 10°C.



------- Linear regression correlation coefficient, r = 0.982 (plotted for 0 to 12 h data sets only).

Data represent the total number of adhered cells (per cm^2) associated with dental wax surfaces over a 48 h incubation period at 10°C.

Values are the means of bacterial counts made by SEM in ten random areas over the surface of three replicate samples. Bars shown are standard errors of the mean.

significant at the 95% confidence interval. Adhesion levels at 24 and 48 h were not significantly different ($p > 0.1$). The adhesion isotherm was, therefore, described as stabilising from 12 to 24 h and saturated by 24 h.

Experimental parameters for this and the previous experiment (Section 3.4.3.ii.a) were identical with the exception that two different cell batches were used for inoculum. Adhesion levels to dental wax at 3 and 24 h may therefore be compared between experiments, to determine whether significant inter-batch variability exists. Adhesion levels when expressed as a percentage of the total inoculated cells ml^{-1} were (3 h = 0.028%, S.E. = 0.006) and (24 h = 2.671%, S.E. = 0.253) for this current section and (3 h = 0.047%, S.E. = 0.007) and (24 h = 2.074%, S.E. = 0.144) for section 3.4.3.ii.a. These levels were significantly different for $p < 0.1$ (Student's t-test) but not significantly different at the 95% confidence interval. This demonstrates that only a very small inter-batch variability existed between the two experiments at 3 and 24 h.

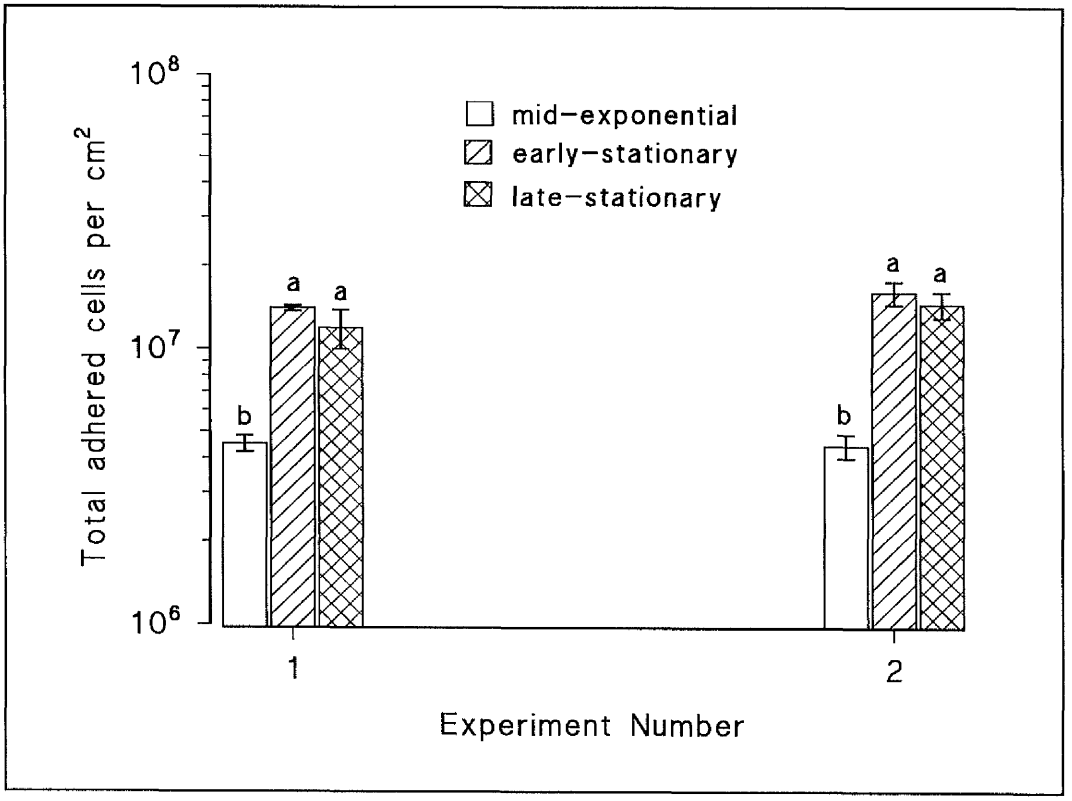
For all further experiments adhesion was measured up to 24 h after inoculation to ensure that adhesion levels had become saturated.

3.5.2 Effect of growth phase of *L. monocytogenes* CRA 433 cells on their adhesion to dental wax surfaces at 10°C.

L. monocytogenes CRA 433 cells were grown in TSB broth at 10°C and collected by centrifugation 40, 72 and 96 h after inoculation. Using a standard growth curve (constructed as detailed in Section 2.4.2 and illustrated in Appendix B), sampling times corresponded to mid-exponential, early-stationary and late-stationary phase cells respectively. Cells were resuspended in 1/4 strength Ringer's solution to an $O.D._{420}$ = 0.56 for inoculation onto dental wax surfaces. Surfaces were analyzed using SEM adhesion analysis 24 h after inoculation. The experiment was performed twice, each time using a different batch of cells. The viable cell count of each inoculum suspension was tested using the spread plate technique on TSA and found to be indistinguishable between the three growth phases (5.38×10^8 cfu ml^{-1}, S.D. = 2.25×10^7 cfu ml^{-1}).

Adhesion levels for both experiments are illustrated in Fig. 3.17. Adhesion levels for early-stationary and late-stationary phase cells were not significantly different from

Figure 3.17 Effect of bacterial growth phase on the adhesion of *L. monocytogenes* CRA 433 to dental wax following 24 h incubation at 10°C.



Data for two replicate experiments (1 & 2) are presented.

For each experiment *L. monocytogenes* CRA 433 was grown in TSB medium at 10°C and collected at mid-exponential, early-stationary and late-stationary growth phases. Cells were resuspended in 1/4 strength Ringer's solution for inoculation onto dental wax and adhesion (per cm^2) quantified following a 24 h incubation period at 10°C.

Values are the means of total bacterial counts made by SEM in ten random regions over the surface of each of three replicate samples $\pm$ standard errors of the mean (bars).

Columns labelled with the same letter are not significantly different from each other ($p > 0.1$, Multiple Range test).

each other ($p > 0.1$), either within or between experiments. Adhesion for mid-exponential phase cells were also not significantly different between replicate experiments, but were significantly lower ($p < 0.05$, Multiple Range test) than all adhesion levels for early- and late-stationary phase cells.

Inoculum for all future experiments used *L. monocytogenes* cells incubated at 10°C to early-stationary phase.

3.5.3 Adhesion of ultraviolet irradiated and viable *L. monocytogenes* CRA 433 cells to dental wax surfaces at 10°C.

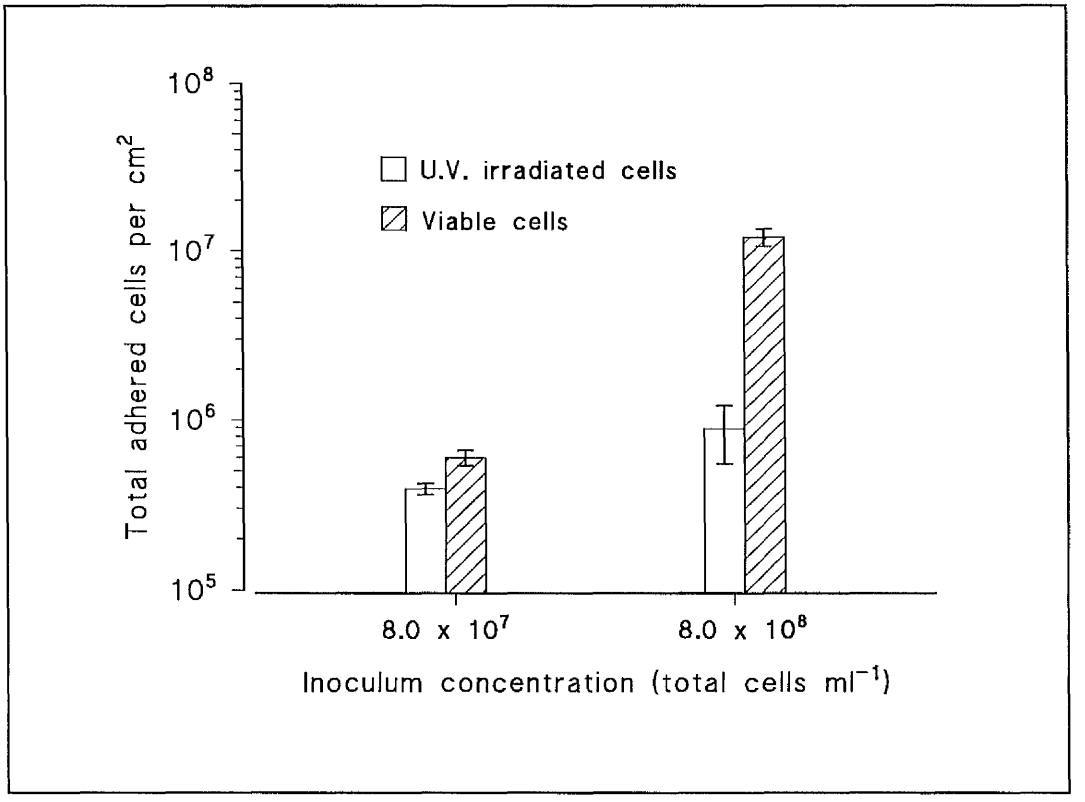
The ability of ultraviolet irradiated (U.V.) *L. monocytogenes* cells to adhere to dental wax was investigated. Viable and U.V. inocula of *L. monocytogenes* CRA 433 cells suspended in 1/4 strength Ringer's solution were prepared as detailed in sections 2.4.3 and 2.4.4 respectively. For both treatments total cell concentrations of 8.0×10^7 cells ml^{-1} (Inoculum A) and 8.0×10^8 cells ml^{-1} (Inoculum B) were prepared from one original cell batch. For both U.V. suspensions, a 2 min irradiation period was sufficient to reduce the viability by 99.99%.

Dental wax surfaces were inoculated with either viable or U.V. cells and adhesion quantified following a 24 h incubation period at 10°C.

Adhesion levels for U.V. and viable *L. monocytogenes* cells are illustrated in Fig. 3.18. Detectable numbers of adhering U.V. irradiated and viable cells were detected for both inoculum concentrations. For both treatments adhesion increased significantly ($p < 0.05$) from inoculum A to B, and was significantly higher for viable than U.V irradiated cells ($p < 0.05$, Student's t-test). The difference in adhesion levels between treatments was smaller at the low inoculum concentration (A) than at the high inoculum concentration (B). For inoculum A, a 1.5 fold difference in adhesion levels between treatments was recorded, whereas for inoculum B, the difference was by a factor of 13.

When adhesion levels were expressed as a percentage of the inoculum concentration, increasing the inoculum concentration resulted in a significant decrease ($p < 0.05$, Student's t-test) in percentage adhesion from 0.488% (S.E. = 0.037) to

Figure 3.18 Adhesion of viable and ultraviolet irradiated *L. monocytogenes* CRA 433 cells to dental wax following 24 h incubation at 10°C.



Data are illustrated for viable and ultraviolet-irradiated (U.V.) cell treatments.

All cells were suspended in 1/4 strength Ringer's solution. Inoculated dental wax surfaces were incubated at 10°C for 24 h.

Values are the means of total bacterial counts made by SEM in ten random regions over the surface of each of three replicate samples ± standard errors of the mean (bars).

0.298% (S.E. = 0.124) for U.V.irradiated cells, and a significant increase ($p<0.05$) from 0.743% (S.E.= 0.077) to 2.112% (S.E. = 0.178) for viable cells.

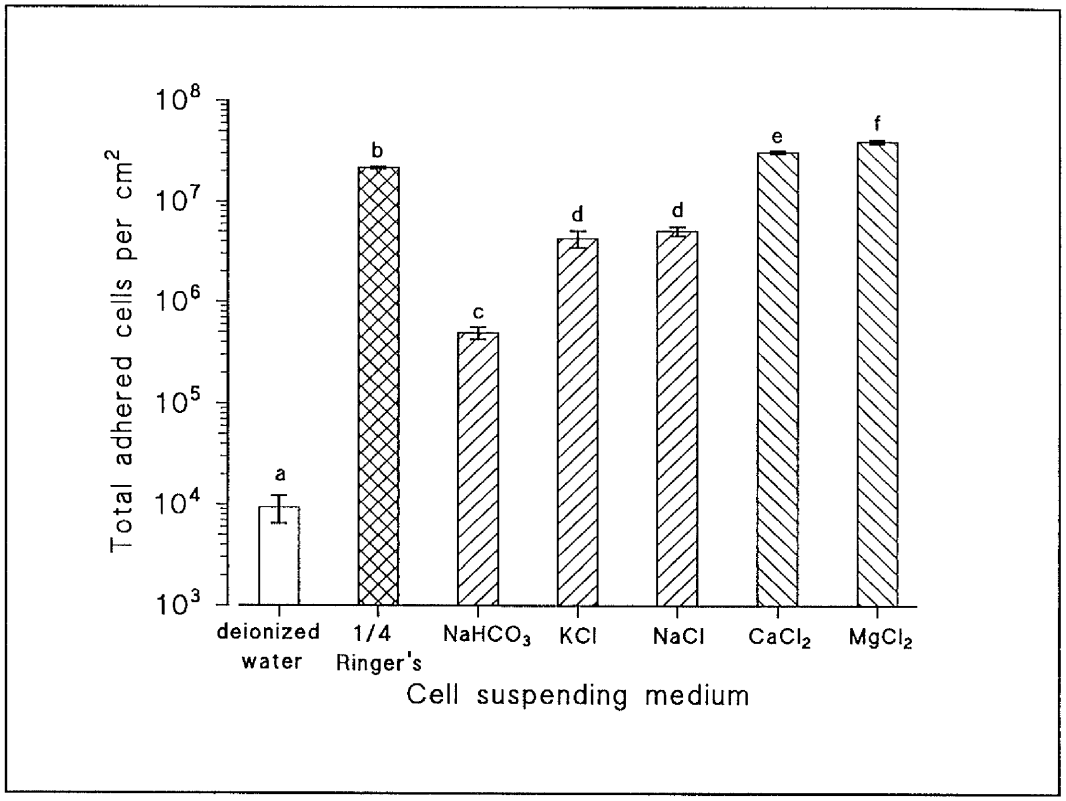
The fall in percentage adhesion levels for U.V. cells, coupled with the relatively small increase in the numbers of adhering cells from inoculum A to B, indicates that the binding isotherm of adhered cells plotted against non-adhering cells was beginning to reach a plateau. In this case, the maximum adhesion level for U.V. cells would not have increased much further if the inoculum concentration had been raised above inoculum B. This may be compared to the situation for viable cells, where both the number of adhering cells and percentage adhesion levels increased from inocula A to B. This indicated that the binding isotherm had not reached a plateau and increasing the inoculum concentration above that of inoculum B would increase the number of cells cm^{-2} on the surface still further. It can be concluded that U.V. irradiated cells were capable of adhesion to dental wax, but the maximum adhesion level (per cm^2) was lower than that of viable cells. For more detailed descriptions of binding isotherms for viable cells see Section 3.6.2 (Fig. 3.25).

3.5.4 The effect of bacterial cell-suspending medium on the adhesion of *L. monocytogenes* to dental wax surfaces at 10°C.

One batch of *L. monocytogenes* CRA 433 cells was divided into seven equal volumes, each volume was washed x3 and resuspended to a total cell concentration of 8.0×10^8 cells ml^{-1} in either deionized water or 40 mM solutions of either the monovalent cations (NaCl, KCl or $NaHCO_3$), divalent cations ($CaCl_2$ or $MgCl_2.6H_2O$) or the mixed cation solution (1/4 strength Ringer's) pH 7.1. Note: 1/4 strength Ringer's is a 40 mM solution comprising of 38.50 mM NaCl, 1.40 mM KCl, 0.59 mM $NaHCO_3$ and 0.55 mM $CaCl_2$. Dental wax surfaces were inoculated and incubated at 10°C for 24 h. Following the standard washing procedure, total adhering cells for each cell-suspending treatment were quantified using SEM adhesion analysis.

Figure 3.19 illustrates adhesion levels on dental wax for each of the seven cell-suspending treatments. Statistical analysis (Multiple Range test) showed that the mean adhesion level for cells suspended in $MgCl_2.6H_2O$ was significantly higher ($p<0.05$)

Figure 3.19 Effect of bacterial cell-suspending medium on the adhesion of *L. monocytogenes* CRA 433 to dental wax following 24 h incubation at 10°C.



Data are presented for seven cell-suspending media. These media fall into four main categories:

□ = No cations (deionized water).

▨ = Only monovalent cations (NaCl, KCl or $NaHCO_3$)

▧ = Only divalent cations ($MgCl_2.6H_2O$ or $CaCl_2$).

▩ = Both monovalent and divalent cations (1/4 strength Ringer's solution)

Values are the means of total bacterial counts made by SEM in ten random regions over the surface of each of three replicate samples ± standard errors of the mean (bars).

Columns labelled with the same letter are not significantly different from each other ($p > 0.05$, Multiple Range test).

than adhesion for cells suspended in $CaCl_2$. These were both significantly higher ($p < 0.05$) than adhesion for cells suspended in 1/4 strength Ringer's solution. Mean adhesion levels for NaCl and KCl were not significantly different from each other ($p > 0.05$), but were significantly lower ($p < 0.05$) than adhesion levels for 1/4 strength Ringer's solution and significantly higher ($p < 0.05$) than $NaHCO_3$. Adhesion for deionized water was significantly lower ($p < 0.05$) than that for the six salt solutions. A 4000 fold difference in adhesion levels between deionized water and $MgCl_2.6H_2O$ was observed.

Plate 3.20.a-g, illustrate SEM micrographs of dental wax surfaces with the range of increasing *L. monocytogenes* adhesion levels described in Fig.3.19. In Plate 3.20.a, an adhesion level of 4.5×10^4 cells cm^{-2} is illustrated; this corresponds to an adhesion level approximately 4 times higher than the mean adhesion level calculated for deionized water. Plate 3.20.b & c illustrate the mean adhesion levels of 5.0×10^5 cells cm^{-2} calculated for $NaHCO_3$, and 5.0×10^6 cells cm^{-2} calculated for NaCl and/or KCl respectively. Plate 3.20.d & e, illustrate adhesion levels of 1.0×10^7 and 2.0×10^7 cells cm^{-2} respectively, corresponding to the lowest and mean adhesion levels calculated for 1/4 strength Ringer's solution. Plate 3.20.f & g, illustrate mean adhesion levels for $CaCl_2$ (3.0×10^7 cells cm^{-2}) and $MgCl_2.6H_2O$ (4.0×10^7 cells cm^{-2}) respectively.

In Plate 3.20.f & g, characteristic circular patterns of adhering *Listeria* cells are visible on each surface. These patterns were a result of the localisation of adhering cells to the lower valley regions of the surface at high adhesion levels. Adhering cells were seldom observed on the highest peak regions of dental wax surfaces. By examining Plate 3.20.a-g in sequence, several stages in the formation of these circular patterns are evident.

At low adhesion levels, below approximately 5.0×10^5 cells cm^{-2} (Plate 3.20.a & b), adhering cells were localised individually to pocket regions on the surface. At adhesion levels above this, small micro-colonies of adhering cells were observed in each pocket region (Plate 3.20.c). These micro-colonies became larger (Plate 3.20.d) and eventually, as the number of cells in each colony increased, adjacent micro-colonies became connected along the valley regions of the surface (Plate 3.20.e). Eventually as

Plate 3.20 Scanning electron micrographs of dental wax surfaces illustrating *L. monocytogenes* adhesion in different electrolyte solutions.

Seven micrographs are presented (a-g), each represents one randomly selected area on a dental wax surface inoculated with *L. monocytogenes* cells.

The number of adhering bacterial cells visible in each micrograph is presented (expressed as cells cm^{-2} of dental wax surface).

Plate 3.20.a illustrates a 8800 μm^2 area of dental wax. The number of bacterial cells visible is equal to an adhesion level 4 times higher than the mean adhesion level calculated for cells suspended in deionized water (presented in Fig. 3.19).

Plate 3.20.b-g illustrate 2200 μm^2 areas of dental wax.

Plate 3.20.b, c, e, f & g illustrate mean adhesion levels (presented in Fig. 3.19) for $NaHCO_3$, NaCl/KCl, 1/4 strength Ringer's solution, $CaCl_2$ and $MgCl_2.6H_2O$ respectively.

Plate 3.20.d illustrates the lowest adhesion level observed for cells suspended in 1/4 strength Ringer's solution.

Bacteria (B) occur individually for the low adhesion levels shown in (Plate 3.20.a & b). Micro-colonies (M) of adhering cells are visible for intermediate adhesion levels (Plate 3.20.c & d). At high adhesion levels (Plate 3.20.f & g) circular patterns of connecting micro-colonies are visible along the valley (V) regions of the surfaces. Bacteria are absent on the peak regions (P) of all surfaces shown.

Plate 3.20.a. A 4.5 x 10^4 cells cm^{-2} adhesion level. This is approximately four times higher than the mean adhesion level for cells suspended in deionized water.

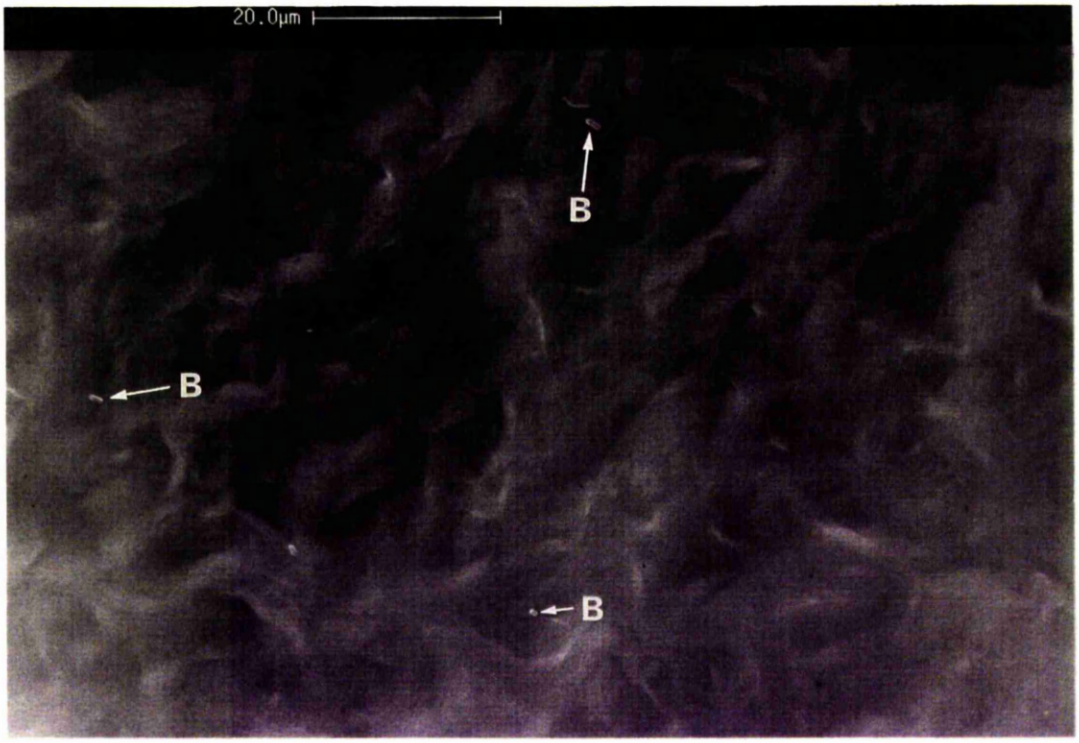


Plate 3.20.b. **A 5.0 x 10^5 cells cm^{-2} mean adhesion level for $NaHCO_3$.**

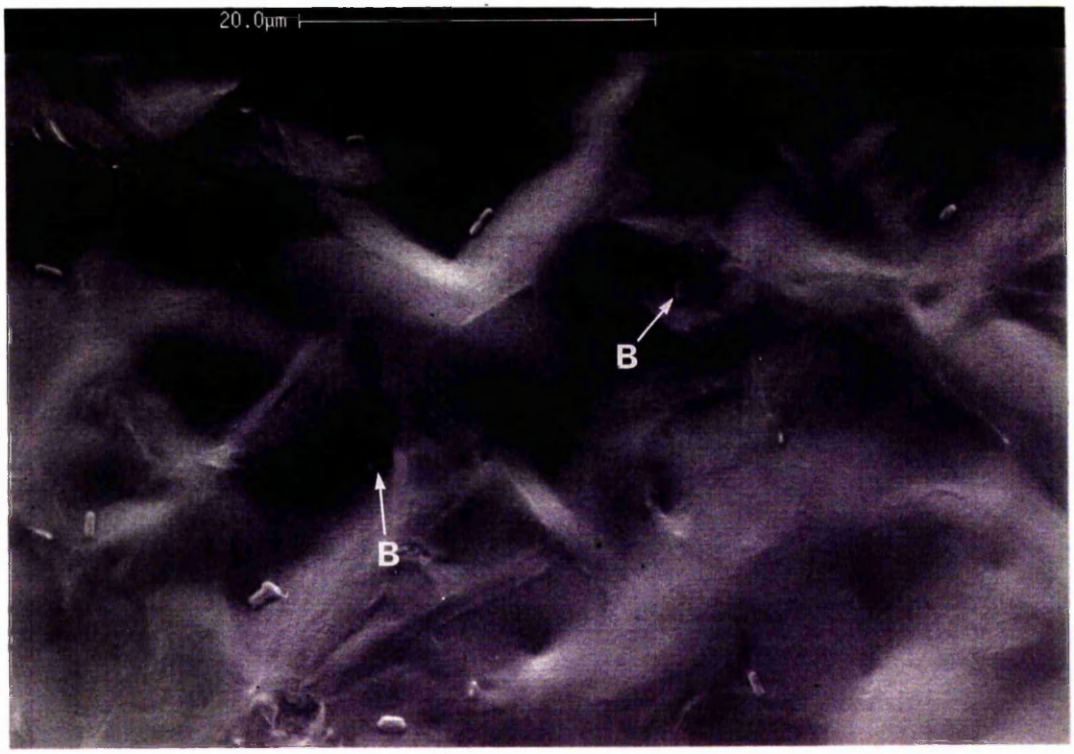


Plate 3.20.c. **A 5.0 x 10^6 cells cm^{-2} mean adhesion level for NaCl and KCl.**

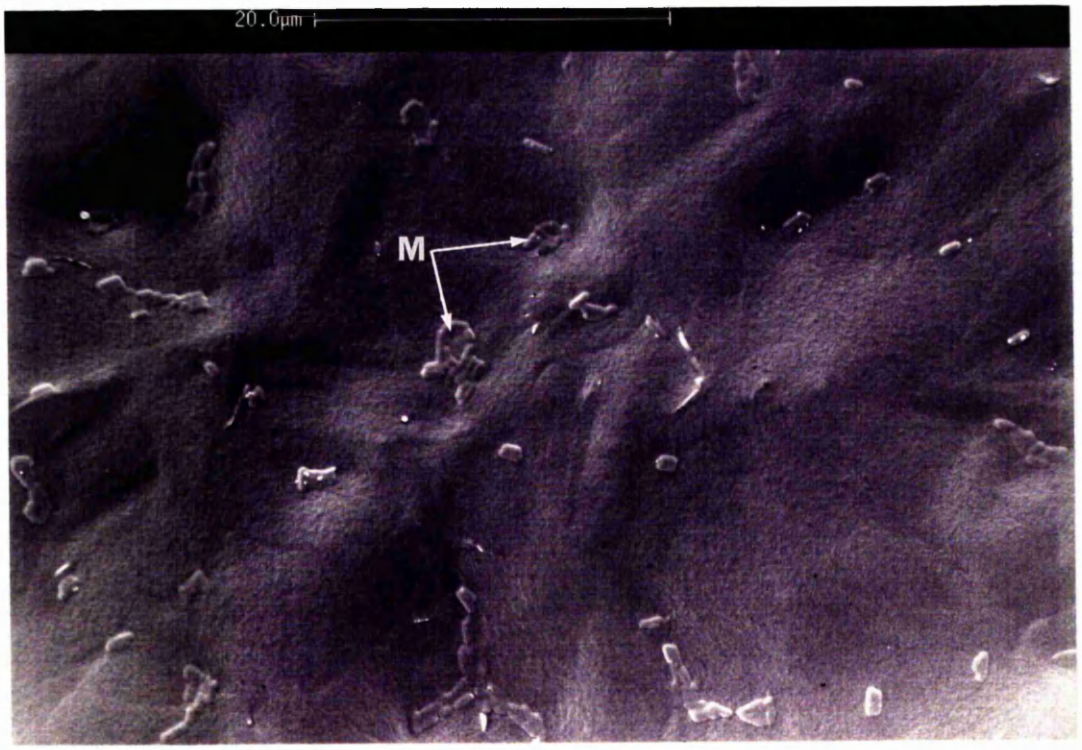


Plate 3.20.d. **A 1.0 x 10^7 cells cm^{-2} lower adhesion level for 1/4 Ringer's.**

Plate 3.20.e. **A 2.0 x 10^7 cells cm^{-2} mean adhesion level for 1/4 Ringer's.**

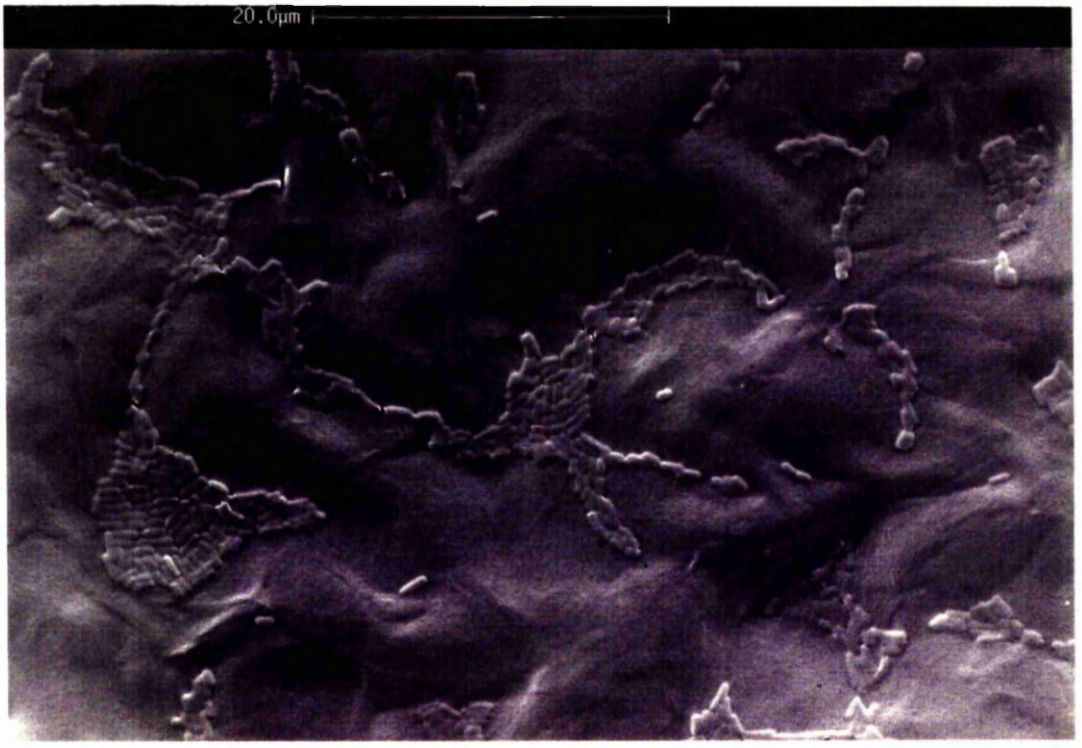


Plate 3.20.f. A 3.0 x 10^7 cells cm^{-2} mean adhesion level for $CaCl_2$.

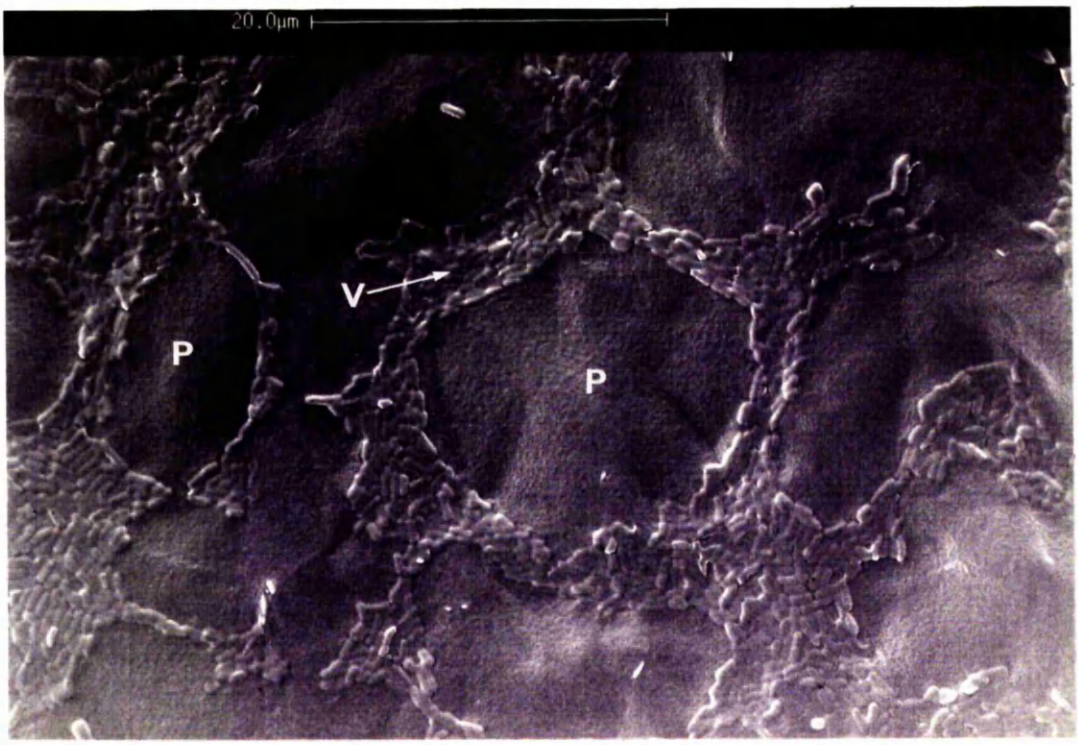
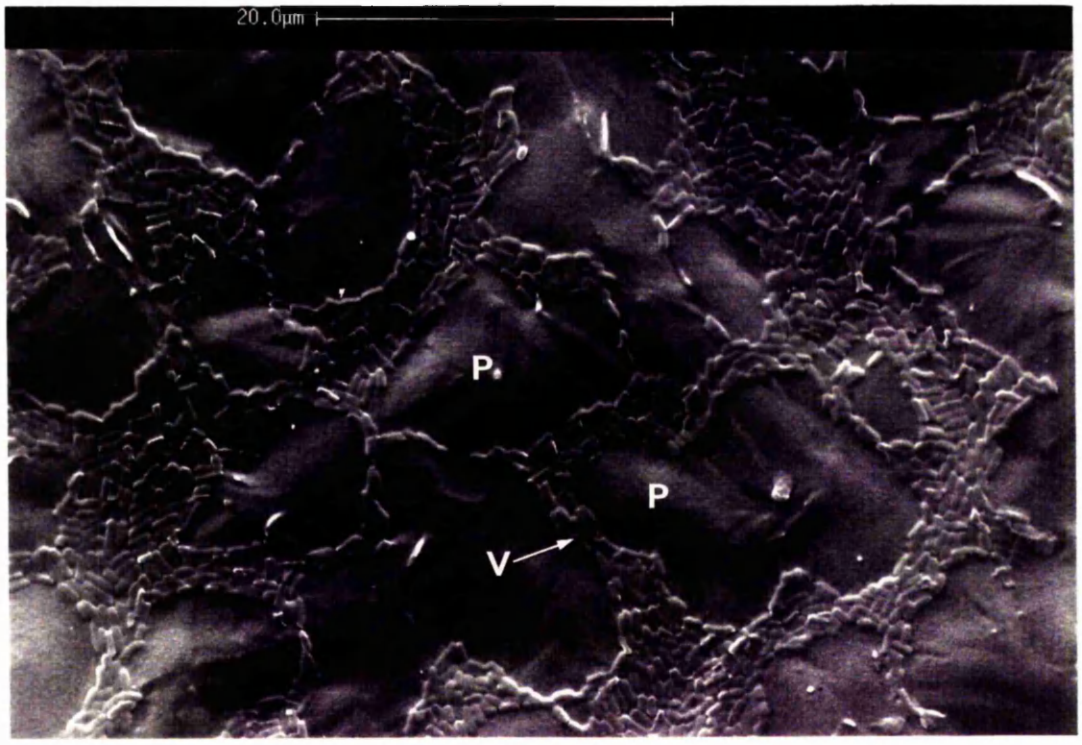


Plate 3.20.g. A 4.0 x 10^7 cells cm^{-2} mean adhesion level for $MgCl_2.6H_2O$



more valley regions were filled, all adjacent micro-colonies met and consequently circular patterns on the surface were formed (Plate 3.20.f). Adhesion increased from this level only if cells could remain adhered to the surface at a point relatively high on the valley to peak transect (Plate 3.20.g).

In this and the previous four experiments examining the adhesion of *L. monocytogenes* CRA 433 to dental wax (Sections 3.4.3.ii.a to Section 3.5.3), mean percentage adhesion levels at 24 h for viable early-stationary phase cells suspended in 1/4 strength Ringer's solution to a total cell concentration of 8.0×10^8 cells ml^{-1} may be compared. These adhesion levels were equal to 2.074% S.E.= 0.144 (Sections 3.4.3.ii.a, Table 3.13), 2.671% S.E.= 0.144 (Section 3.5.1, 24 h), 2.034% S.E = 0.192 (Section 3.5.2), 2.112% S.E. 0.178 (Section 3.5.3, viable inoculum B) and 2.691% S.E.= 0.079 (this present section, Table 3.14). Therefore, the range of mean percentage adhesion levels calculated in the five experiments is from 2.034% to 2.691%. This range corresponds to adhesion levels of between 1.627×10^7 and 2.153×10^7 cells cm^{-2}. Although these are significantly different ($p=0.03$), in practice, the difference when quantifying these two adhesion levels from SEM images showing a 2200 μm^2 area of dental wax (e.g Plate 3.20.b-g) is 115 cells per area. The difference in the number of cells present in Plate 3.20.d & e is almost twice this value at 205 cells. This illustrates, in real terms, the small difference in mean adhesion levels between the five experiments and supports the inter-batch reproducibility of the SEM adhesion assay.

Table 3.14 gives mean percentage adhesion levels for the seven cell-suspending treatments studied in this section. These adhesion levels may be compared to those presented in Tables 3.5 and 3.6 (Section 3.2.4.ii) for cabbage and lettuce leaf tissue respectively (day 1 data only). For cells suspended in deionized water, adhesion levels of 0.02% (cabbage) and 0.052% (lettuce) were both significantly higher ($p<0.05$) than that for dental wax (0.0012%) which was very low. For cells suspended in 1/4 strength Ringer's solution, an adhesion level of 4.513% (cabbage) was significantly higher than the 2.691% adhesion level for dental wax, which in turn, was approximately 10 times higher than the 0.26% adhesion level for lettuce tissue. The difference in adhesion

Table 3.14 Effect of cell-suspending medium on the adhesion of *L. monocytogenes* to dental wax following incubation at 10°C for 24 h.

Cell Suspending Medium	% Adhesion
deionized water	0.0012 ± 0.0004^{a}
40 mM $NaHCO_3$	0.062 ± 0.009^{b}
40 mM NaCl	0.586 ± 0.084^{c}
40 mM KCl	0.535 ± 0.104^{c}
1/4 strength Ringer's	2.691 ± 0.079^{d}
40 mM $CaCl_2$	3.868 ± 0.139^{e}
40 mM $MgCl_2.6H_2O$	4.935 ± 0.225^{f}

Data represent the numbers of total adhered cells recorded at 24 h after inoculation, expressed as a percentage of the total cells inoculated onto the surface at 0 h.

Values are the average of total bacterial counts made from ten SEM micrographs on each of three or four(*) replicate samples ± standard errors of the mean.

Values followed by the same letter are not significantly different from each other ($p > 0.05$, Multiple Range test).

levels between the monovalent cation NaCl and the divalent cation $MgCl_2.6H_2O$ was larger for cabbage tissue than for dental wax. For cabbage tissue, adhesion levels of 2.989% (NaCl) and 6.619% ($MgCl_2.6H_2O$) were both significantly higher than comparative adhesion levels of 0.586% (NaCl) and 4.935% ($MgCl_2.6H_2O$) for dental wax.

3.5.5 Adhesion of *L. monocytogenes* CRA 433 to dental wax for cells suspended in different Molar salt solutions.

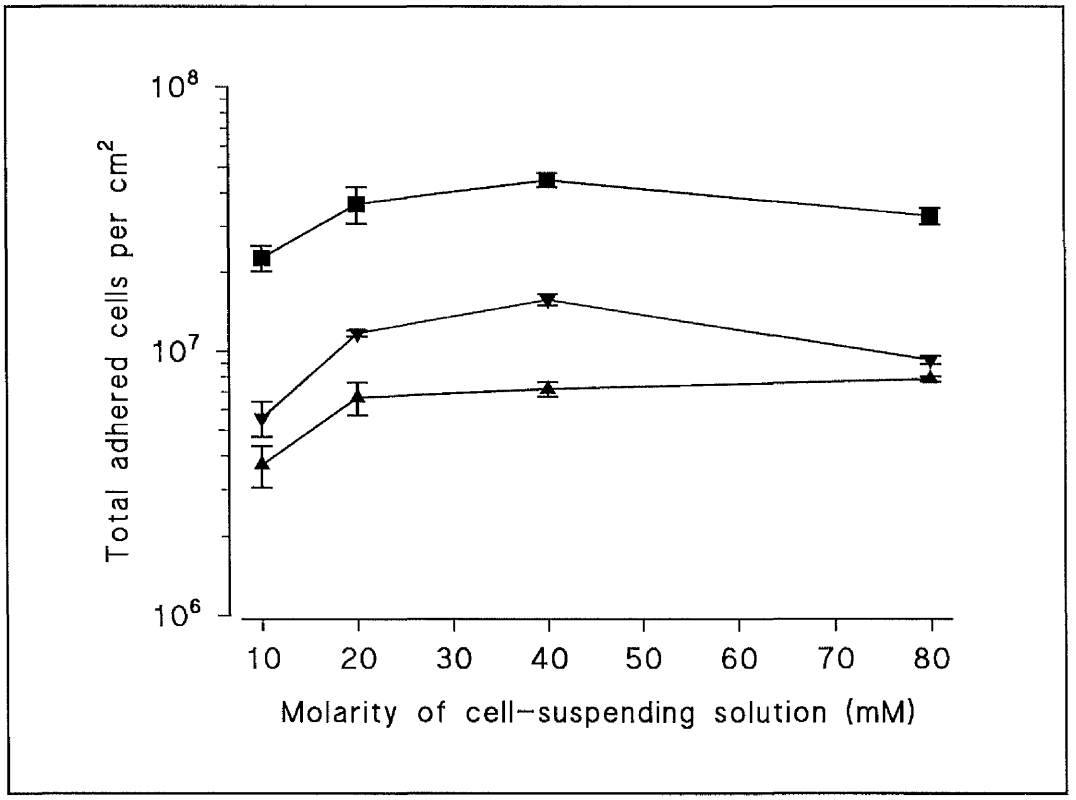
One batch of *L. monocytogenes* CRA 433 cells grown in TSB was divided into three equal volumes and each washed x3 in distilled water. Cells were resuspended to a concentration of 8.0×10^8 cells ml^{-1} in either 10, 20, 40, or 80 mM solutions of NaCl, $MgCl_2.6H_2O$ or Ringer's solution. Note: 10, 20, 40 and 80 mM Ringer's concentrations are equal to 1/16, 1/8, 1/4 and 1/2 strength Ringer's solution.

Dental wax surfaces were inoculated with one cell suspension and adhering *L. monocytogenes* cells quantified using SEM adhesion analysis following a 24 h incubation period at 10°C.

Adhesion levels for the three cell-suspending treatments are illustrated in Fig. 3.20. At 10 mM concentrations, adhesion levels for NaCl and Ringer's solution were not significantly different ($p > 0.1$, Student's t-test). At all other concentrations tested, adhesion was significantly higher in solutions of Ringer's than in NaCl. At all molar concentrations, adhesion was significantly higher ($p < 0.05$, Multiple Range test) for cells suspended in $MgCl_2.6H_2O$ than in both NaCl and Ringer's solutions.

Adhesion increased significantly ($p < 0.05$) from 10 mM to 20 mM NaCl, but was not significantly different above a 20 mM concentration ($p > 0.1$, Multiple Range test). For both Ringer's and $MgCl_2.6H_2O$ suspending treatments, adhesion increased significantly from 10 mM to maximum adhesion levels at 40 mM concentrations and then fell significantly ($p < 0.05$) above 40 mM concentrations.

Figure 3.20 Adhesion of *L. monocytogenes* CRA 433 to dental wax at 24 h when suspended in different molar concentrations of three salt solutions.



Cell-suspending solutions.

■ = $MgCl_2.6H_2O$

▼ = Ringer's (Strength: 1/16 = 10 mM, 1/8 = 20 mM, 1/4 = 40 mM, 1/2 = 80 mM)

▲ = NaCl

Values are the means of total bacterial counts made by SEM in ten random regions over the surface of each of three replicate samples ± standard errors of the mean (bars).

3.5.6 Adhesion to dental wax of four *L. monocytogenes* isolates. Correlation between adhesion levels and cell-surface hydrophobicity.

The cell surface hydrophobicity of four *L. monocytogenes* isolates, CRA 433 4b, CRA 5246 4b, ATCC 23074 4b and F139 1/2a, has previously been determined under different salt environments using phase separation with n-hexadecane (Section 3.3.2.i.b, Table 3.8). The adhesion to dental wax of the original cell batches of these four isolates was measured using SEM adhesion analysis (Section 3.5.6.i). For isolate CRA 433, adhesion was also measured for a partitioned cell batch containing only hydrophilic cells and compared with a cell batch (of equal concentration) containing a mixed population of hydrophilic and hydrophobic cells in a 10:1 ratio (Section 3.5.6.ii).

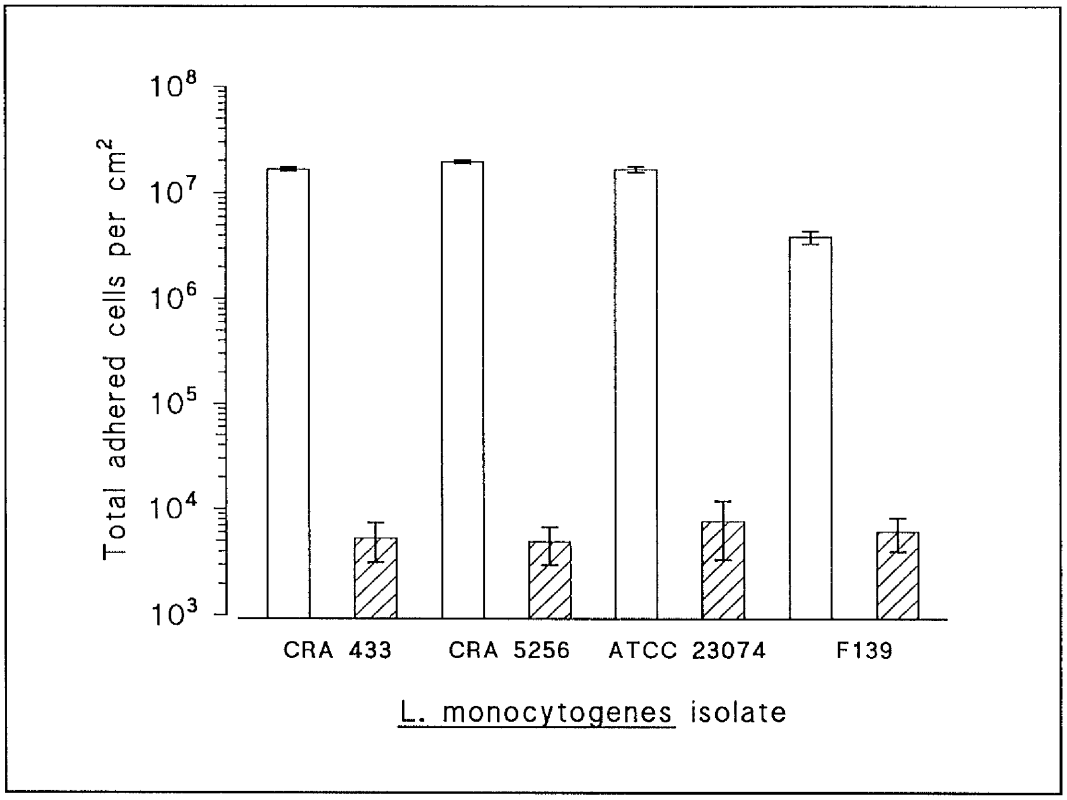
3.5.6.i Adhesion of four un-partitioned *L. monocytogenes* isolates to dental wax when suspended in 1/4 strength Ringers solution and deionized water.

For adhesion analysis of the four *L. monocytogenes* isolates detailed above, the $O.D._{420}$ of the original (un-partitioned) cell suspensions were lowered by 9.26% (from $O.D._{420} = 0.6$ to $O.D._{420} = 0.555$) using either 1/4 strength Ringer's solution or deionized water. Dental wax surfaces were inoculated with either isolate and analyzed using SEM adhesion analysis 24 h after incubation at 10°C.

Figure 3.21 illustrates adhesion levels for the four *L. monocytogenes* isolates when suspended in 1/4 strength Ringer's solution and deionized water. No significant differences in adhesion levels ($p > 0.1$, ANOVA) were found between isolates when the cells were suspended in deionized water. Average hydrophobicity values of approximately 2% in deionized water were also not significantly different between the four isolates (Section 3.3.2.i.b, Table 3.8).

Both the adhesion levels (Fig. 3.21) and hydrophobicity values (Table 3.8) for the four *L. monocytogenes* isolates increased when cells were suspended in 1/4 strength Ringer's solution compared to deionized water. Increases in adhesion were by factors of approximately 634 for isolate F139 and 3150 for isolates CRA 433, CRA 5246 and ATCC 23074. Adhesion levels for the latter three isolates were not significantly different, but were all obviously higher than that for isolate F139. Hydrophobicity

Figure 3.21 Adhesion of four *L. monocytogenes* isolates to dental wax at 24 h when suspended in deionized water and 1/4 strength Ringer's solution.



Cell-suspending solutions.

▨ = deionized water

□ = 1/4 strength Ringer's solution

Data represents adhesion levels for *L. monocytogenes* isolates CRA 433 4b, CRA 5246 4b, ATCC 23074 4b and F139 1/2a in two cell-suspending solutions.

Values are the means of total bacterial counts made by SEM in ten random regions over the surface of each of three replicate samples ± standard errors of the mean (bars).

values also increased significantly from approximately 2% in deionised water to 3.37% (F139), 7.7% (ATCC 23074), 9.26% (CRA 433) and 9.31% (CRA 5246) in 1/4 strength Ringer's solution (Table 3.8).

Because isolate F139 was serotype 1/2a whilst isolates ATCC 23074, CRA 433 and CRA 5246 were serotype 4b, the possibility existed that the differences in adhesion levels observed were due to serotype variation. For this reason the study was extended to measure adhesion levels to dental wax for nine *L. monocytogenes* isolates including four different serotypes (see Section 3.6.3).

3.5.6.ii Adhesion to dental wax surfaces for a 100% hydrophilic and 91% hydrophilic *L. monocytogenes* CRA 433 cell population of equal concentration.

In order to determine if only hydrophobic cells were capable of adhering to dental wax surfaces, hydrophobic cells of *L. monocytogenes* CRA 433 were removed from the inoculum (by partitioning with n-hexadecane) and adhesion compared to the original un-partitioned population at an equal cell concentration. The previous experiment (Section 3.5.6.i) measured the adhesion of the original un-partitioned cell population of $O.D._{420}$ = 0.555 containing 9.26% hydrophobic cells and 90.74% hydrophilic cells in 1/4 strength Ringer's solution. For comparison, the 100% hydrophilic population was obtained by partitioning the original cell suspension with n-hexadecane from $O.D._{420}$ = 0.6 to $O.D._{420}$ = 0.555 (9.26% drop in $O.D._{420}$ due to the removal of hydrophobic cells). Adhesion to dental wax for the 100% hydrophilic and 91% hydrophilic population was then compared using SEM adhesion analysis.

No significant difference in adhesion levels between the 100% hydrophilic (1.57×10^7 cells cm^{-2}, S.E. = 1.57×10^6) and 91% hydrophilic (1.66×10^7 cells cm^{-2}, S.E. = 7.4×10^5) cell populations were observed ($p > 0.1$, Student's t-test). This shows that hydrophilic cells which do not partition into n-hexadecane are still capable of adhering to dental wax.

3.6 Adhesion of *L. monocytogenes* to dental wax; quantification using photometric adhesion analysis of crystal violet stained cells.

3.6.1 Calibration of photometric adhesion assay.

The very high levels of adhesion (up to approximately 5.0 x 10^7 cells cm^{-2}) on dental wax surfaces observed in Section 3.5.4, were very difficult and time consuming to measure using SEM adhesion analysis. A rapid screening adhesion assay was therefore developed. Using the protocol detailed in Section 2.12, the new assay used crystal-violet staining of adhering *L. monocytogenes* cells on dental wax surfaces. Stain intensity, measured using optical density ($O.D._{570}$) readings, was calibrated against total bacterial counts in the stained film. Future experiments could then transform values of $O.D._{570}$ directly into numbers of adhered bacteria.

Initially, a two stage calibration experiment was performed to determine the correlation between the $O.D._{570}$ of a crystal-violet stained *L. monocytogenes* film and the total cell count in the film. The two stages of the experiment differed principally in the way the count of bacterial cells was made. In stage 1 (detailed fully in Section 3.6.1.i) a range of *L. monocytogenes* total cell concentrations were prepared using a Helber counting chamber. Each concentration was then dried onto the dental wax surface, fixed and stained to produce a range of $O.D._{570}$ values. In stage 2 (described fully in Section 3.6.1.ii), a range of stained bacterial films were selected from stage 1, viewed under SEM, and a total count of *L. monocytogenes* cells within each film was made using the previously developed SEM adhesion analysis protocol.

3.6.1.i. Correlation between the concentration of *L. monocytogenes* CRA 433 cells dried onto dental wax surfaces and the $O.D_{570}$ of the stained bacterial film produced (Stage 1).

Acetate discs coated on one side with a thin layer of dental wax were prepared (Section 2.12.1). Each disc was screened for uniformity (Section 2.12.2) and those having an $O.D._{570}$ of 0.28 $\pm$ 0.003 were selected for inoculation (Note: an $O.D._{570}$ = 0.28 was the average absorbency for 1000 acetate discs coated on one side with dental

wax). *L. monocytogenes* cells were suspended in a 40 mM solution of $MgCl_2.6H_20$ at the six total cell concentrations shown in Table 3.15. Each cell concentration was used to inoculate ten replicate dental wax discs as described in Section 2.12.3. The inoculum was dried fully onto the wax surface by placing the discs for 30 min under vacuum at room temperature. Bacterial films were then fixed and stained with crystal violet (Section 2.12.5). Note: bacterial films were only washed following the fixation procedure so as not to remove any dried cells from the surface. Average $O.D._{570}$ values for the dried bacterial films were then calculated against un-inoculated control discs (Section 2.12.6). In this instance, control discs were inoculated with a 40 mM solution of $MgCl_2.6H_2O$ without the addition of cells. The average $O.D._{570}$ of ten replicate bacterial films was then plotted against their corresponding initial inoculum concentration (Fig. 3.22).

Figure 3.22 illustrates that the $O.D._{570}$ of the bacterial film was linearly related to the number of cells originally dried onto the surface. The linear regression correlation coefficient, r = 0.993. This excellent correlation was characterised by the linear regression line described by equation 3.1:

$(y) = 6.146 \times 10^{-9} (x) - 0.027351$ (equation 3.1)

where: (y) = $O.D._{570}$ of the bacterial film and (x) = number of *L. monocytogenes* cells per cm^2 dried onto the surface.

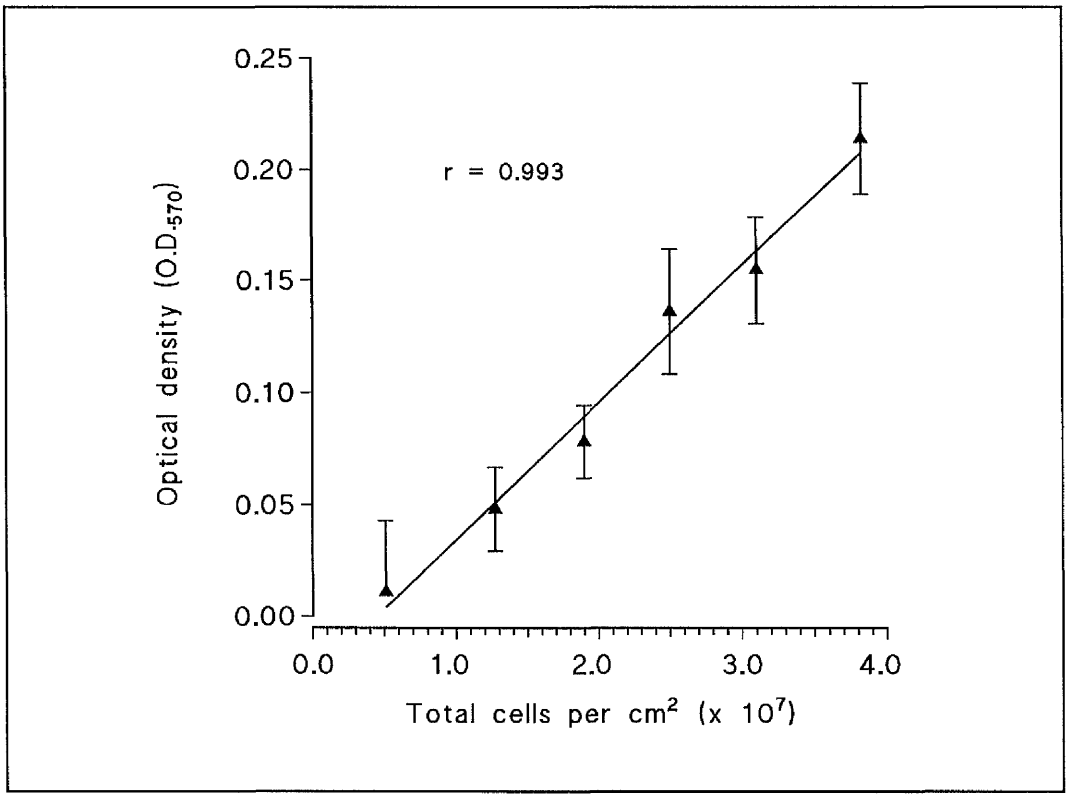
Table 3.15 Total *L. monocytogenes* CRA 433 cell concentrations dried onto dental wax surfaces in the Stage 1 photometric calibration experiment.

Total cells ml^{-1}	Total cells dried per cm^2 of dental wax surface
2.0×10^7	5.10×10^6
5.0×10^7	1.28×10^7
7.45×10^7	1.90×10^7
1.0×10^8	2.55×10^7
1.22×10^8	3.11×10^7
1.5×10^8	3.83×10^7

Data illustrate the total cell counts (per ml) of *L. monocytogenes* CRA 433 cells for six inocula. Drops, 50 μl of inocula were dried onto 0.196 cm^2 areas of dental wax.

Total cell counts were made using a Helber counting chamber.

Figure 3.22 Correlation between the total *L. monocytogenes* CRA 433 cell concentration dried onto dental wax surfaces, and the optical density $O.D._{570}$ of the crystal-violet stained bacterial film. Stage 1 calibration experiment.



———— Linear regression correlation coefficient, r = 0.993

Data illustrate the optical density $O.D._{570}$ of stained bacterial films plotted against the total concentration of cells dried onto the surface. Total cell concentrations were calculated using a Helber counting chamber (Table 3.15).

Values are the means of $O.D._{570}$ readings for ten replicate dental wax surfaces. Bars shown are standard deviations.

3.6.1.ii. Correlation between the $O.D._{570}$ of a stained *L. monocytogenes* film on dental wax and the total number of cells in the film as determined from SEM analysis (Stage 2).

From stage 1, individual dental wax discs having bacterial film $O.D._{570}$ of 0.01, 0.020, 0.022, 0.027, 0.036, 0.044, 0.047, 0.074, 0.076, 0.084, 0.13, 0.132, 0.148, 0.151, 0.157, 0.214, 0.221 and 0.232 were selected, mounted onto SEM stubs and sputter coated with gold as described in Section 2.7.4.iii. Total bacterial counts in ten random regions over each bacterial film were then made using the SEM adhesion analysis (Section 2.11.2.ii). The average total bacterial count per cm^2 for each film was then plotted against its corresponding initial $O.D._{570}$ value (Fig. 3.23).

Figure 3.23 illustrates that the total count of *L. monocytogenes* cells in the stained film was linearly related to the optical density $O.D._{570}$ of bacterial film. The linear regression correlation coefficient, r=0.984. This good correlation was characterised by the best fit regression line described by equation 3.2:

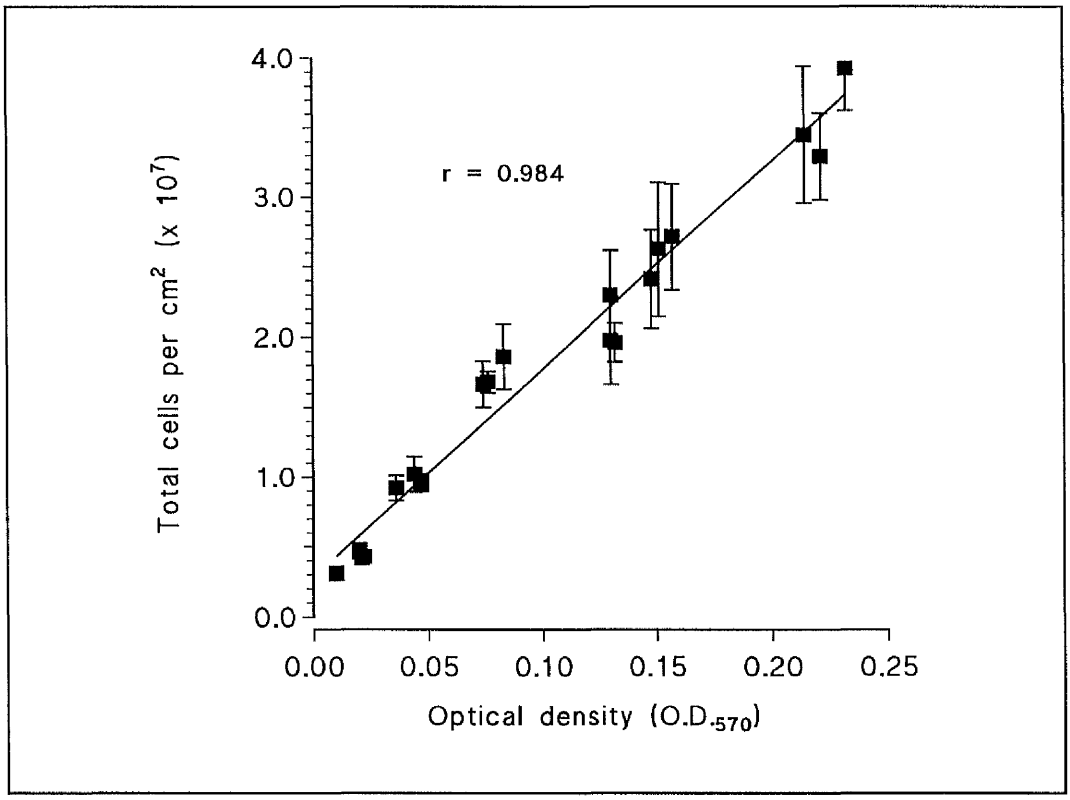
$(y) = 1.489 \times 10^8 (x) + 2.833 \times 10^6$ (equation 3.2)

where: (y) = total number of *L. monocytogenes* cells per cm^2 and, (x) = $O.D._{570}$ of the bacterial film.

3.6.1.iii Correlation between data obtained in Stage 1 and Stage 2 photometric calibration experiments.

Figure 3.24 illustrates data obtained from stages 1 & 2 of the calibration experiment described above (presented seperately in Figs. 3.22 and 3.23 respectively). Data is plotted with total cells per cm^2 as the unknown variable (i.e. on the y-axis). For this reason standard deviations for stage 1 are not shown, as they were variations in $O.D._{570}$ of the bacterial film and not cell numbers. From Fig. 3.24 it is clear that there was a good correlation between the two stages of the experiment; data for stage 1 lies within the 95% confidence interval of stage 2 data sets. The graphs were slightly divergent at high cell concentration levels. This possibly indicates that in stage 1, as the inoculum concentration increased, a slightly larger proportion of the dried cells in the

Figure 3.23 **Correlation between the optical density $O.D._{570}$ of a crystal-violet stained bacterial film, and the total bacterial count of cells in the film as measured using SEM adhesion analysis. Stage 2 calibration experiment.**

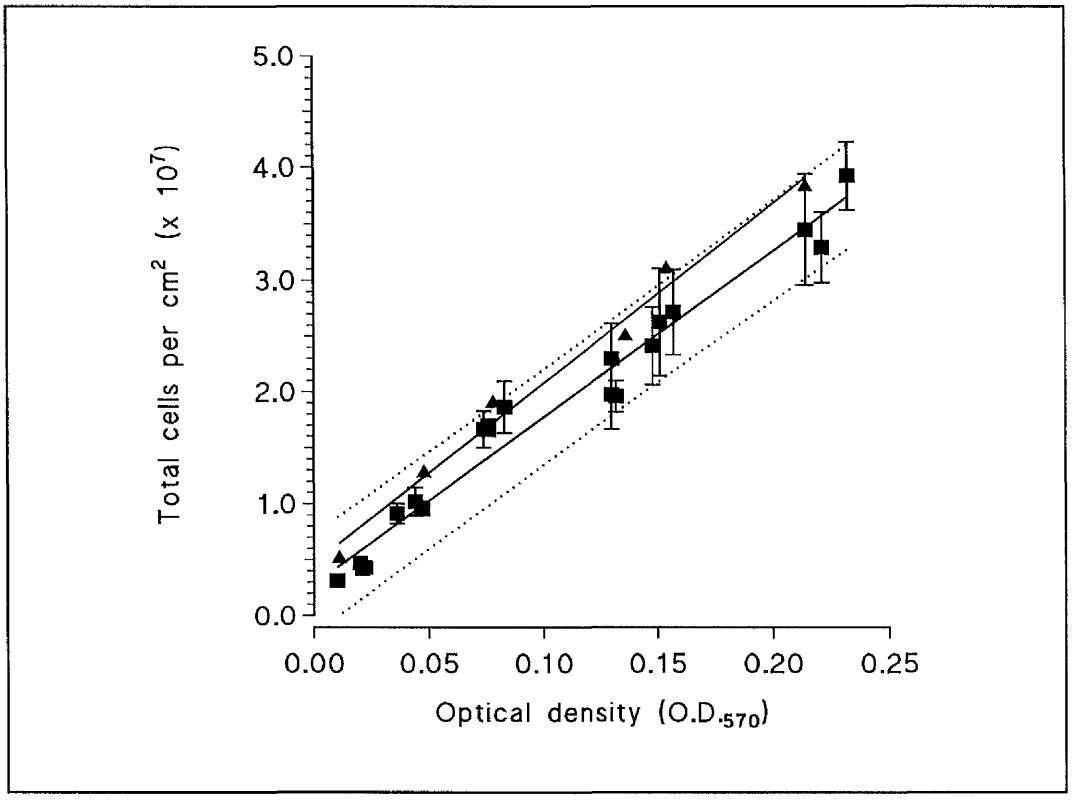


——— Linear regression correlation coefficient, r = 0.984

Data illustrate the total bacterial cell counts made using SEM adhesion analysis over the surface of stained bacterial films of known $O.D._{570}$.

Values are the means of total bacterial counts made by SEM in ten random regions over the surface of each stained bacterial film. Bars shown are standard deviations.

Figure 3.24 Correlation between stage 1 and stage 2 photometric calibration experiments.



Experiments.

▲ = Stage 1. Data for bacterial film optical density $O.D._{570}$ was obtained following the drying of a known total cell concentration (by Helber chamber counts) onto dental wax surfaces.

Values are the means of $O.D._{570}$ readings for ten replicate dental wax surfaces. Errors are not shown because they were deviations in $O.D._{570}$ and not cell number.

■ = Stage 2. Data illustrates the total bacterial cell counts made using SEM adhesion analysis over the surface of stained bacterial films of known $O.D._{570}$, from stage 1.

Values are the means of total bacterial counts made by SEM in ten random regions over the surface of each stained bacterial film. Bars shown are standard deviations.

------- 95% confidence limits of stage 2.

film became detached from the surface. This would probably have occurred during the fixation procedure and consequently the actual number of cells in the stained bacterial film was lower than the initial cell concentration plotted.

For future experiments, where $O.D._{570}$ values of stained *L. monocytogenes* populations were transformed into total numbers of cells per cm^2 of surface, equation 3.2 was used in preference to equation 3.1. This was because two experimental errors were present in stage 1. Firstly, and as already mentioned above, the initial cell concentration dried onto the dental wax surface was probably higher than the actual number of cells which remained on the surface following the fixation and staining procedures. This was not a problem in stage 2, because the total cell counts were made directly from the dental wax surface following all specimen preparation. Secondly in stage 1, at $O.D._{570}$ values of below 0.048 only one data point (0.011 ± 0.016) was obtained. Because of the large standard deviation of this data point, the correlation equation (equation 3.1) which was derived from it could not be relied upon. In addition, if this data point was omitted from the correlation this would result in the minimum detection level of the adhesion assay being raised, unacceptably, from approximately 5.0×10^6 to 1.3×10^7 cells cm^{-2}. In stage 2, seven specimens all having $O.D._{570}$ values below 0.0148 were examined (the lowest having an $O.D._{570} = 0.01 \pm 0.001$) and total bacterial counts made from each. Consequently a much better representation of adhesion levels for samples having low optical densities was made in stage 2 compared to stage 1. The minimum detection level of the adhesion assay was, therefore, much lower and more reliable in stage 2 than in stage 1. Using equation 3.2, the minimum detection level for a bacterial film $O.D._{570} = 0.01$ corresponded to a total bacterial count of 4.3×10^6 cells cm^{-2}.

For further adhesion experiments, if the average optical density $O.D._{570}$ for ten replicate samples was ≤ 0.015 (slightly higher than the minimum detection level of the adhesion assay) then the three replicates having bacterial film $O.D._{570}$ closest to 0.015 were selected, prepared for SEM examination, and total bacterial counts per cm^2 calculated for each using SEM adhesion analysis. In this way both high levels ($\geq 5.07 \times 10^6$ cells cm^{-2}, corresponding $O.D._{570} = 0.015$) and low levels ($\leq 5.07 \times 10^6$ cells

cm^{-2}) of adhering bacteria could be assessed rapidly using photometric analysis and SEM adhesion analysis respectively.

It should be noted that where photometric analysis is performed in future experiments, cells were inoculated onto a surface area of dental wax = 0.196 cm^2. This was a smaller area compared to 0.785 cm^2 used in Sections 3.4.3 to 3.5.6. The drop size of the cell inoculum was, therefore, also smaller in all future experiments (50 μl compared to 500 μl). This may have lowered adhesion levels slightly due to an increase in surface tension forces within the drop. Additionally adhesion levels may be slightly lower due to the additional fixation and staining procedures involved in the photometric adhesion assay.

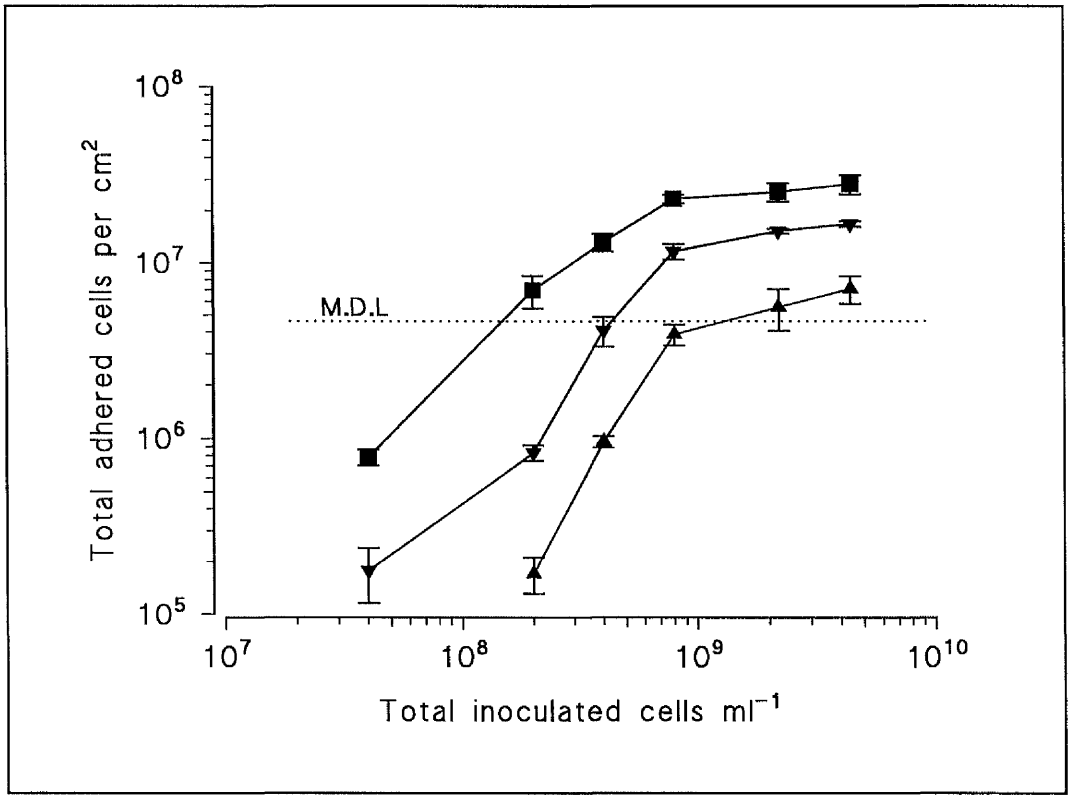
3.6.2 Effect of *L. monocytogenes* population level on their adhesion to dental wax at 10°C when suspended in different salt solutions.

In Section 3.2.3, the adhesion of *L. monocytogenes* CRA 433 cells to lettuce leaf tissue was examined using a range of inoculum concentrations. Binding isotherms and Scatchard plots of adhesion data were plotted to investigate saturation effects and possible cooperative adhesion mechanisms for cells suspended in 1/4 strength Ringer's solution. Saturation was observed at inoculum concentrations of between 8.0 x 10^7 cells ml^{-1} and 8.0 x 10^8 cells ml^{-1}. Positive cooperativity was demonstrated up to intermediate adhering cell densities when possible negative cooperative effects were seen.

In this current section, a similar experiment to that described in Section 3.2.3 was performed on dental wax surfaces. *L. monocytogenes* CRA 433 cells were grown in TSB media and resuspended in either 1/4 strength Ringer's solution or 40 mM solutions of $MgCl_2.6H_20$ or NaCl at each of six total cell concentrations between 4.0 x 10^7 and 4.4 x 10^9 cells ml^{-1} measured by Helber chamber counts. Each cell concentration was used to inoculate ten replicate dental wax surfaces each with 50 μl volumes. Following incubation at 10°C for 24 h, surfaces were prepared for photometric adhesion analysis and SEM adhesion analysis where appropriate.

Binding isotherms of adhesion data for each cell-suspending medium are illustrated in Fig. 3.25. Using statistical analysis (Multiple Range test) adhesion at all

Figure 3.25 Binding isotherms to dental wax surfaces at 24 h for *L. monocytogenes* CRA 433 cells suspended in three different salt solutions.



Cell-suspending media.

■ = $MgCl_2.6H_2O$ (40 mM solution)

▲ = NaCl (40 mM solution)

▼ = 1/4 strength Ringer's solution

------ M.D.L (minimum detection level of photometric assay = 5.07 x 10^6 cells cm^{-2}).

Values below the M.D.L were calculated using SEM adhesion analysis, total bacterial counts were made in ten random regions over the surface of each of three replicate samples ± standard errors of the mean (bars).

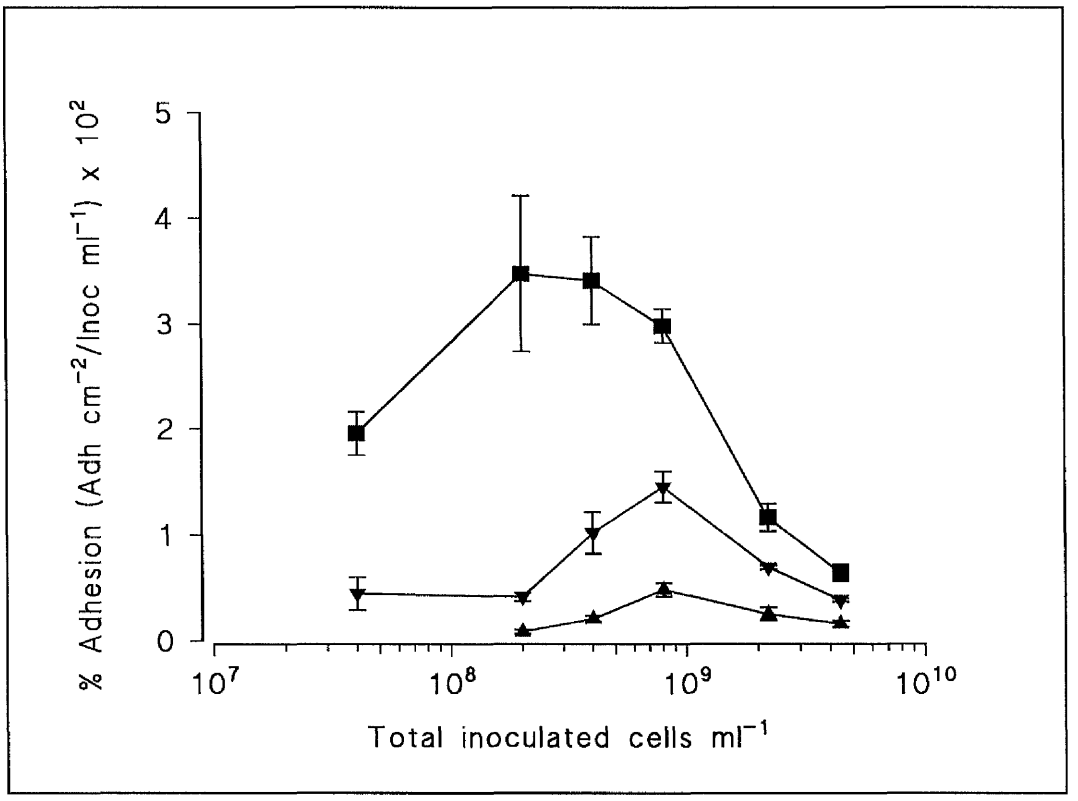
Values above the M.D.L were calculated using photometric adhesion analysis for ten replicate samples ± standard errors of the mean (bars).

inoculum concentrations was significantly higher ($p < 0.05$) for cells suspended in $MgCl_2.6H_2O$ than 1/4 strength Ringer's solution, which in turn was significantly higher ($p < 0.05$) than adhesion of cells suspended in NaCl. At the highest inoculum concentration studied (4.4×10^9 cells ml^{-1}), the adhesion level for NaCl was approximately 25% that of the adhesion level for $MgCl_2.6H_2O$. This percentage gradually decreased with decreasing inoculum concentration, and at a concentration of 2.0×10^8 cells ml^{-1} was calculated as 2.5%. This decrease is also illustrated by the divergence in the binding isotherms of $MgCl_2.6H_2O$ and NaCl at low inoculum concentrations as adhesion for NaCl fell steeply. If the difference between adhesion levels at each inoculum concentration was constant then both isotherms would be parallel. The gradual decrease from 25% to 2.5% indicates that the strength of adhesion decreased at a faster rate for NaCl than $MgCl_2.6H_2O$ suspended cells with decreasing inoculum concentration.

Saturation of available adhesion sites on the substratum is reached when the number of adhered cells is insensitive to an increase in the number of cells in suspension (i.e. when binding isotherms begin to plateau Fig. 3.25). Adhesion levels did not increase significantly ($p > 0.05$) above inoculum concentrations of 8.0×10^8 cells ml^{-1} for $MgCl_2.6H_2O$, and 2.2×10^9 cells ml^{-1} for 1/4 strength Ringer's solution and NaCl (Fig. 3.25). Saturation, therefore, occurred at a slightly lower inoculum concentration for $MgCl_2.6H_2O$ than the other two salt solutions. This is also observed in Fig. 3.26, where percentage adhesion levels for the three media are plotted against inoculum concentration; each graph increases as saturation is approached, and then decreases following saturation. Since all isotherms did reach saturation, this indicates that the inoculum concentration was not restricting adhesion for NaCl and 1/4 strength Ringer's suspended cells to values below that measured for $MgCl_2.6H_2O$. In other words, adhesion for NaCl and 1/4 strength Ringer's solution would not reach that observed for $MgCl_2$ irrespective of any further increases in inoculum concentration.

Figure 3.27.a-c illustrates Scatchard plots of adhesion data for $MgCl_2.6H_2O$, 1/4 strength Ringer's solution and NaCl suspended cells respectively. The ratio of adhered to planktonic cells is plotted against the number of adhered cells. As with lettuce leaf

Figure 3.26 Percentage adhesion levels to dental wax after 24 h for *L. monocytogenes* CRA 433 cells suspended in three salt solutions at a range of cell population levels.



Data represent the number of total adhered cells (per cm^2) 24 h after inoculation expressed as a percentage of the total cells (per ml) inoculated onto the surface at 0 h. Data for three cell-suspending media over a range of cell inoculum concentrations are presented.

Cell-suspending media.

■ = $MgCl_2.6H_2O$ (40 mM solution).

▲ = NaCl (40 mM solution).

▼ = 1/4 strength Ringer's solution.

Bars shown are standard errors of the mean.

Figure 3.27 Scatchard plots of adhered *L. monocytogenes* cells on dental wax surfaces for three cell-suspending media.

The adhering to planktonic cell ratio is plotted against the adhering cell population, at 24 h, for three cell-suspending treatments.

a) Cells suspended in 40 mM $MgCl_2.6H_2O$.

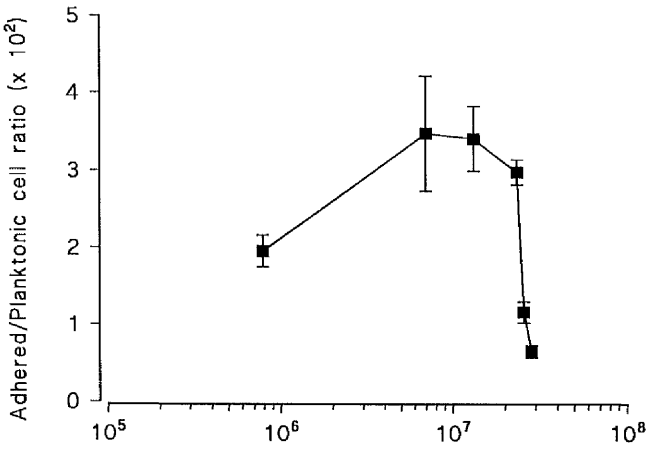
b) Cells suspended in 1/4 strength Ringer's solution.

c) Cells suspended in 40 mM NaCl.

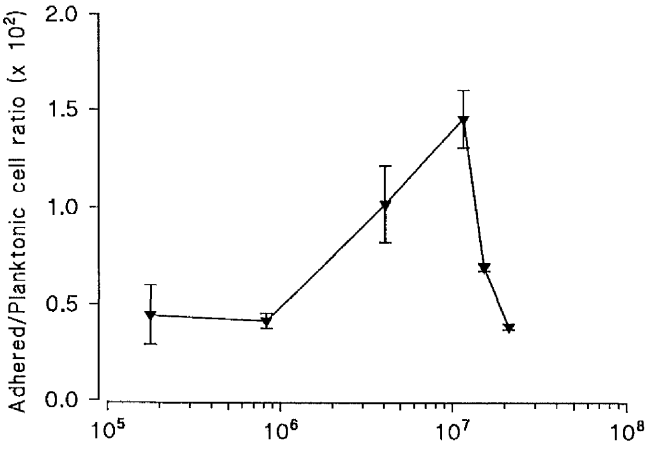
Data are calculated as the number of total adhered cells detected per cm^2 of dental wax surface divided by the total number of cells ml^{-1} in planktonic suspension.

Bars shown are standard errors of the mean.

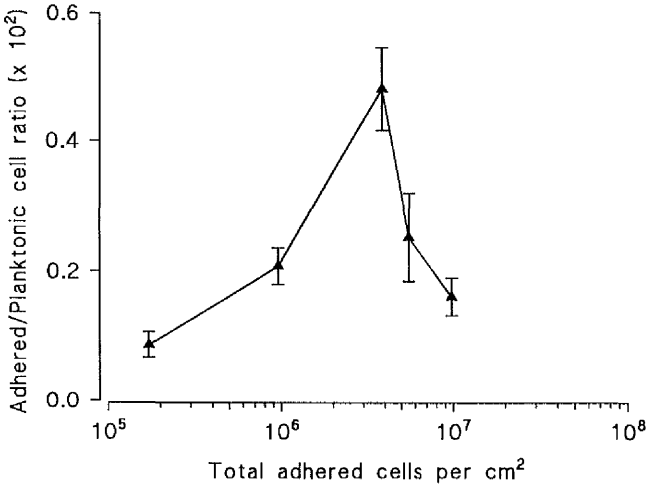
a)


Adhered/Planktonic cell ratio (x 10²)
5
4
3
2
1
0
10⁵
10⁶
10⁷
10⁸

b)


Adhered/Planktonic cell ratio (x 10²)
2.0
1.5
1.0
0.5
0.0
10⁵
10⁶
10⁷
10⁸

c)


Adhered/Planktonic cell ratio (x 10²)
0.6
0.4
0.2
0.0
10⁵
10⁶
10⁷
10⁸
Total adhered cells per cm²

tissue (Section 3.2.3), positive cooperativity between adhering cells was demonstrated for each cell-suspending medium. This is illustrated by the positive upward slope of each graph. Above intermediate adhering cell densities, all graphs demonstrated a negative downward slope. The turning point from positive to negative cooperativity was at slightly higher binding concentrations for $MgCl_2.6H_2O$ (1.32×10^7 cells cm^{-2}) than for 1/4 strength Ringer's solution (1.16×10^7 cells cm^{-2}) and subsequently than that for NaCl (3.86×10^6 cells cm^{-2}).

Percentage adhesion levels at the 8.0×10^8 cells ml^{-1} inoculum concentration were equal to 2.984% S.E. = 0.163 ($MgCl_2.6H_2O$), 1.455% S.E. = 0.148 (1/4 strength Ringer's solution, and 0.482% S.E. = 0.063 (NaCl). These may be compared to those presented in Section 3.5.4 (Table 3.14) for $MgCl_2.6H_2O$ (4.935%), 1/4 strength Ringer's solution (2.691%) and NaCl (0.586%) which were measured on dental wax using SEM analysis. From this comparison between experiments, significant differences for $MgCl_2.6H_2O$ and 1/4 strength Ringer's solution are evident.

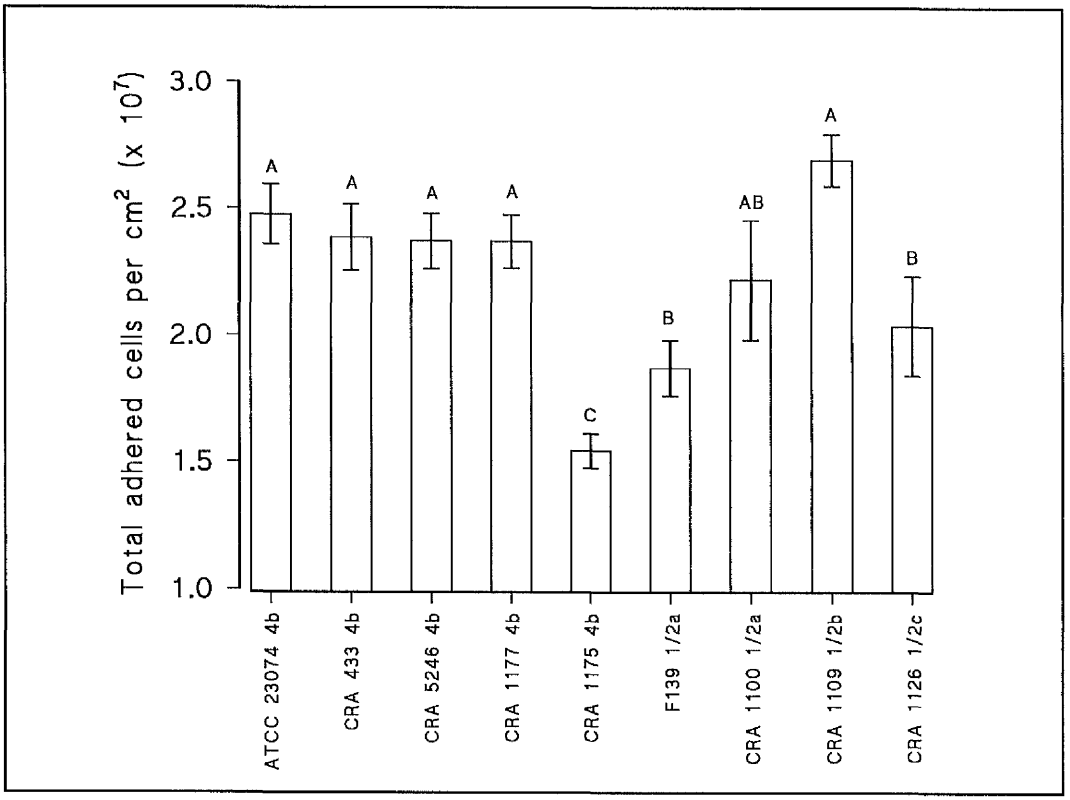
3.6.3 Adhesion of nine *L. monocytogenes* isolates to dental wax when suspended in 40 mM solutions of $MgCl_2.6H_2O$.

L. monocytogenes isolates CRA 433, CRA 5246, CRA 1175, CRA 1177 and ATCC 23074 (serotype 4b), F139 (serotype 1/2a), CRA 1100 (serotype 1/2a), CRA 1109 (serotype 1/2b) and CRA 1126 (serotype 1/2c) were grown in TSB at 10°C until early-stationary phase. Cells were resuspended in 40 mM solutions of $MgCl_2.6H_2O$ to a total cell concentration of 8.0×10^8 cells ml^{-1} and dental wax surfaces inoculated.

Figure 3.28 illustrates adhesion levels measured using photometric adhesion analysis of surfaces following a 24 h incubation period at 10°C. Small but significant variation in adhesion levels between isolates was observed, mean adhesion levels ranged from 1.54×10^7 cells cm^{-2} (S.E. = 6.8×10^5) for isolate CRA 1175 up to 2.69×10^7 cells cm^{-2} (S.E. = 1.0×10^6) for isolate CRA 1109.

Interestingly, and as previously observed in Section 3.5.6.i, the adhesion level for isolate F139 1/2a was significantly lower ($p < 0.05$, Multiple Range test) than that of isolates ATCC 23074, CRA 433 and CRA 5246 (all 4b isolates) which were not

Figure 3.28 Adhesion of nine *L. monocytogenes* isolates to dental wax at 24 h when suspended in 40 mM solutions of $MgCl_2.6H_2O$.



Data represent adhesion levels to dental wax at 24 h for nine *L. monocytogenes* isolates suspended in 40 mM solutions of $MgCl_2.6H_2O$.

Values were calculated using photometric analysis of ten replicate samples $\pm$ standard errors of the mean (bars).

Columns labelled with the same letter are not significantly different from each other ($p > 0.05$, Multiple Range test).

significantly different from each other. The lower adhesion level for isolate F139 was not, however, the result of the difference in serotypes between these four strains. This may be concluded because the adhesion of isolate CRA 1175 4b was significantly lower than all four above mentioned isolates (Fig. 3.28).

Percentage adhesion levels for isolates ATCC 23074, CRA 433 and CRA 5246 were not significantly different ($p > 0.1$, ANOVA) to those measured for isolate CRA 433 (2.984%) in the previous section (a total cell inoculum concentration of 8.0×10^8 cells ml^{-1} was used in both experiments). This, therefore, demonstrates an excellent inter-batch reproducibility of the photometric adhesion analysis protocol.

3.6.4 Importance of cell deposition on the adhesion of *L. monocytogenes* to dental wax following a 24 h incubation period at 10°C.

L. monocytogenes CRA 433 cells were suspended in a 40 mM solution of $MgCl_2.6H_2O$ to a total cell concentration of 8.0×10^8 cells ml^{-1} as described previously. Dental wax discs were inoculated in one of three ways.

i) A 50 μl drop was deposited onto the wax surface (conventional method where cells sediment down onto the wax surface over a 24 h incubation period = control treatment).

ii) A 50 μl drop was placed onto the surface of a plastic petri-dish and a disc floated, wax surface downwards, on the surface of the drop (bacterial cells would sediment towards the petri dish base and away from the wax surface.

iii) Dental wax surfaces were held face downwards at an angle of 45° to the horizontal and in contact with a 50 μl drop of bacterial suspension.

All surfaces were washed after a 24 h incubation period and then prepared for photometric examination as described previously.

The adhesion level for the control treatment of 2.92×10^7 cells cm^{-2} (S.E. 1.31×10^6) was not significantly different to those measured in the previous two experiments (Sections 3.6.2 & 3.6.3). For the second and third inoculation protocols described

above, adhesion levels of 7.44 x 10^6 cells cm^{-2} (S.E. = 1.32 x 10^6) and 8.17 x 10^6 cells cm^{-2} (S.E. = 1.2 x 10^6) respectively were significantly lower ($p > 0.05$) than the control treatment but not significantly different from each other ($p > 0.1$). Although this data is very limited, it does suggest that sedimentation plays an important role in the adhesion of *L. monocytogenes* to dental wax. The observation that cells were still capable of adhesion without sedimentation may indicate that additional mechanisms were involved in the initial approach of the bacteria to the surface. The control inoculation protocol was used for all further experiments.

3.6.5 Reinoculation of dental wax surfaces already saturated with adhering *L. monocytogenes* cells.

At two staggered time points with a 24 h period between each, two batches of early-stationary *L. monocytogenes* cells were prepared in TSB medium. Both cell batches were resuspended in 1/4 strength Ringer's solution to a total cell concentration of 2.2 x 10^9 cells ml^{-1} to produce two inoculum suspensions (I1 & I2). This cell concentration corresponded to the saturation concentration for 1/4 strength Ringer's solution determined in section 3.6.2. Six batches of ten dental wax discs were incubated, washed and in some cases reinoculated using one of the six protocols described below.

i) I1 was deposited on to dental wax surfaces which were then washed (10 dips in distilled water) following a 24 h incubation period at 10°C.

ii) I2 was deposited on to dental wax surfaces which were then washed (10 dips in distilled water) following a 24 h incubation period at 10°C.

iii) I1 was deposited on to dental wax surfaces and the surface washed (10 dips in distilled water) following a 48 h incubation period at 10°C.

iv) I1 was deposited on to dental wax surfaces which were then washed (10 dips in distilled water) following a 24 h incubation period at 10°C. Surfaces were then re-incubated for a further 24 h at 10°C.

v) I1 was deposited on to dental wax surfaces and the surface washed twice (20 dips in

distilled water) following a 48 h incubation period at 10°C

vi) I1 was deposited on to dental wax surfaces, washed (10 dips in distilled water) following a 24 h incubation period, air dried for 10 min and reinoculated using I2. Surfaces were then re-incubated for a further 24 h at 10°C and again washed (10 dips in distilled water).

Following all procedures dental wax discs were prepared for photometric adhesion analysis.

The schematic diagram illustrated in Fig. 3.29 shows how the six protocols, detailed above, were used in four separate adhesion experiments. Each experiment is described separately below. Adhesion levels for the six protocols are illustrated in Fig. 3.30. Adhesion levels for protocols i to v inclusive were not significantly different from each other ($p > 0.1$). The adhesion level for protocol vi was significantly lower than that of protocol ii ($p < 0.1$, Multiple Range test).

Experiment 1:

Adhesion of two batches of *L. monocytogenes* cells following a 24 h incubation period were compared (comparison between protocols i & ii). Adhesion was not significantly different between the two batches indicating good inter-batch reproducibility.

Experiment 2:

Adhesion of *L. monocytogenes* to dental wax following 24 and 48 h incubation periods were compared (protocols i and iii). In addition, these adhesion levels were compared to those for dental wax surfaces washed at 24 h and re-incubated for a further 24 h (comparison between protocols i, iii and iv). No significant difference between the three protocols was found ($p > 0.1$). Correlation in adhesion between protocols i and iii demonstrates that adhesion did not increase significantly past a 24 h incubation period. Secondly, the correlation between protocols i and iv indicates that following initial adhesion, the bacterial cells did not multiply in numbers on the dental wax surface. The possibility still exists that the cells did divide without a change in cell volume.

Figure 3.29 Schematic diagram illustrating four experiments measuring the adhesion of *L. monocytogenes* to dental wax at 10°C. Surfaces were prepared using six protocols.

I1 = Inoculum suspension 1.
I2 = Inoculum suspension 2.
W = Time point at which dental wax surfaces were washed by dipping in distilled water. (W1 = Washed with 10 dips, W2 = Washed with 20 dips).

Six inoculation protocols were used in four experiments.

Protocols
i) I1 inoculated at 0 h. Surfaces washed (W1) at 24 h.
ii) I2 inoculated at 24 h. Surfaces washed (W1) at 48 h.
iii) I1 inoculated at 0 h. Surfaces washed (W1) at 48 h.
iv) I1 inoculated at 0 h. Surfaces washed (W1) at 24 h. Surfaces then re-incubated up to 48 h.
v) I1 inoculated at 0 h. Surfaces washed (W2) at 48 h.
vi) I1 inoculated at 0 h. Surfaces washed (W1) at 24 h. Surfaces dried and re-inoculated with I2 at 24 h. Surfaces re-incubated up to 48 h and again washed (W1); Total washing = W2.

Experiments
1) Adhesion of I1 and I2 to dental wax following 24 h incubation.
2) Adhesion of I1 to dental wax following a 24 h and 48 h initial incubation. Effect of re-incubation for 24 h (illustrated by arrow) on the adhesion level for samples initially washed at 24 h.
3) Adhesion of I1 following a 48 h incubation period and either 10 or 20 washes in distilled water.
4) Effect on adhesion of re-inoculating a dental wax surface saturated with adhering *L. monocytogenes* cells.

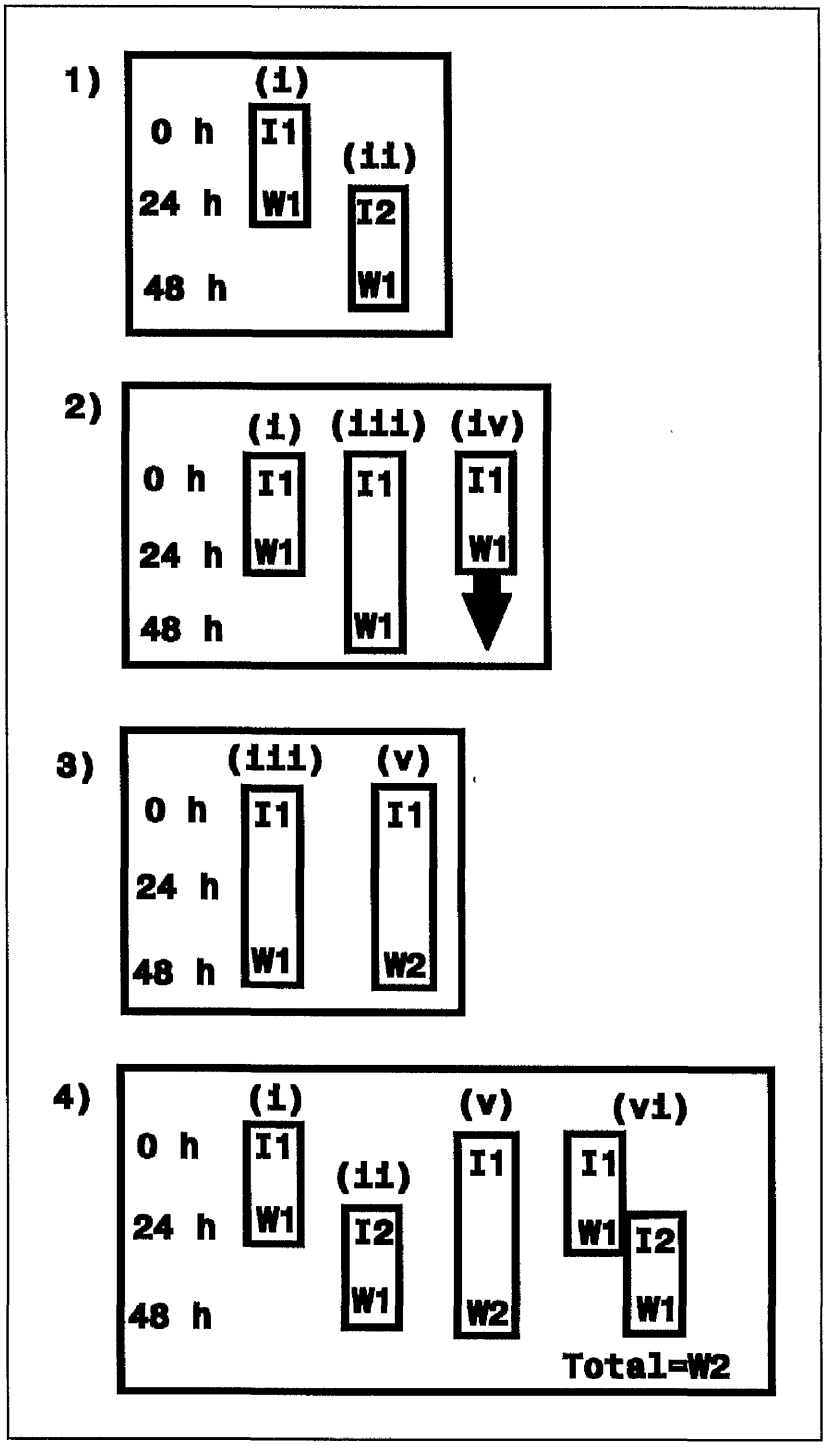
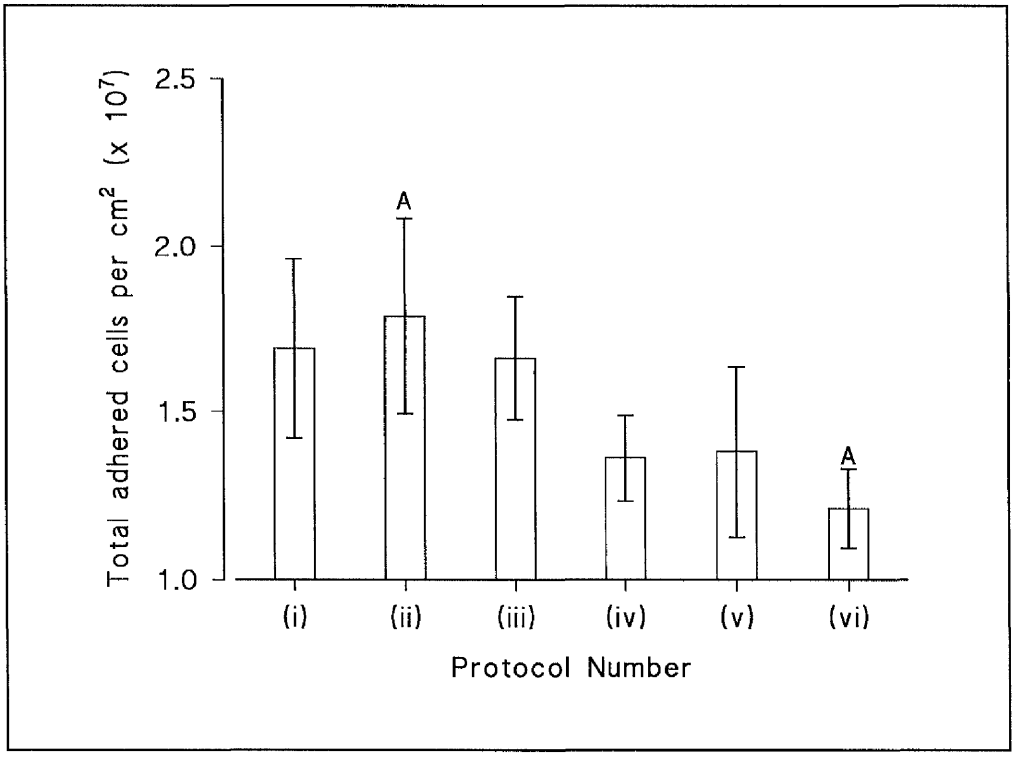

1)
(i)
0 h
I1
W1
(ii)
24 h
I2
W1
48 h
2)
(i)
(iii)
(iv)
0 h
I1
W1
I1
I1
W1
24 h
W1
48 h
3)
(iii)
(v)
0 h
I1
I1
24 h
W1
W2
48 h
4)
(i)
(v)
(vi)
0 h
I1
W1
(ii)
I1
I1
W1
24 h
I2
W1
I2
W1
48 h
W2
Total=W2

Figure 3.30 Adhesion of *L. monocytogenes* CRA 433 to dental wax following either of six inoculation protocols.



Data represent adhesion levels to dental wax under six inoculation protocols.

Two inoculum suspensions were used for inoculation (I1 and I2).
Two washing procedures in distilled water were used. W1 = 10 dips, W2 = 20 dips.

Inoculation protocols.
i) I1 inoculated at 0 h, surfaces washed (W1) at 24 h.
ii) I2 inoculated at 24 h, surfaces washed (W1) at 48 h.
iii) I1 inoculated at 0 h, surfaces washed (W1) at 48 h.
iv) I1 inoculated at 0 h, surfaces washed (W1) at 24 h and re-incubated up to 48 h.
v) I1 inoculated at 0 h, surfaces washed (W2) at 48 h.
vi) I1 inoculated at 0 h, surfaces washed (W1) at 24 h, air dried and reinoculated with I2. Surfaces were re-incubated up to 48 h and again washed (W1). Total washing = W2.

Values were calculated using photometric analysis for ten replicate samples $\pm$ standard errors of the mean (bars).

Columns labelled with the same letter are significantly different from each other ($p<0.1$).

Experiment 3:

The removal of adherent *L. monocytogenes* cells from dental wax surfaces using two washing procedures was assessed. Inoculated surfaces were incubated for 48 h before being washed with either 10 or 20 dips in distilled water (comparison between protocols iii and v). Adhesion levels following the two washing procedures were not significantly different ($p > 0.1$). Doubling the washing procedure did not, therefore, reduce adhesion levels significantly.

Experiment 4:

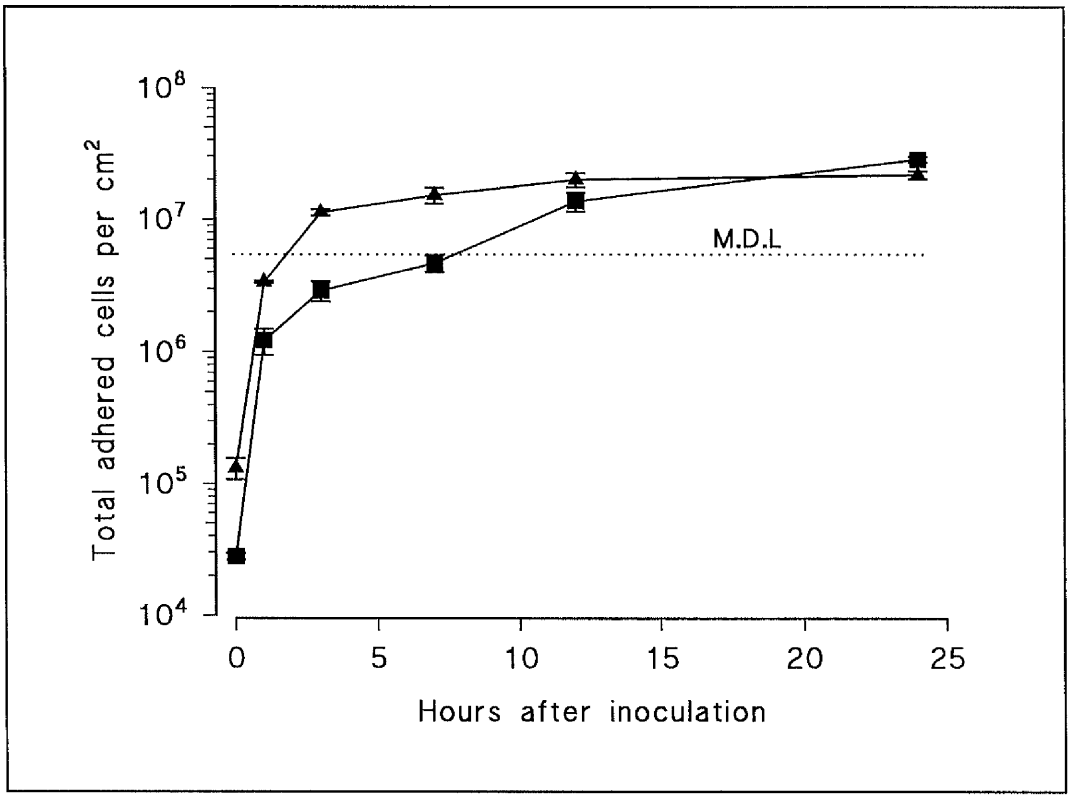
The effect on adhesion of reinoculating dental wax surfaces which had adherent cells already attached to them was investigated (protocol vi). Theoretically, if adhesion levels did not increase following reinoculation it could be concluded that the availability of adhesion sites on the dental wax surface, and not the availability of bacterial cells capable of adhesion, was the factor limiting adhesion. Controls for the experiment principally included protocols i, ii and v and, in addition, protocols iii and iv. As described above, only adhesion levels for protocols ii and vi were significantly different ($p < 0.1$) from each other. Adhesion levels of the reinoculated surface did in fact decrease significantly ($p < 0.1$) from that of a surface which had been inoculated with only I2 (protocol ii).

3.6.6 Adhesion of *L. monocytogenes* CRA 433 to plasma-glow discharged dental wax surfaces over a 24 h incubation period at 10°C.

Plasma-glow discharged dental wax (PGD) was prepared (Section 2.11.1.vb). Adhesion of *L. monocytogenes* cells resuspended in 40 mM $MgCl_2.6H_2O$ over a 24 h incubation period was compared for PGD and untreated dental wax surfaces using photometric and SEM adhesion analysis where appropriate.

Figure 3.31 illustrates adhesion levels to PGD and untreated dental wax surfaces over the 24 h incubation period. Adhesion to PGD surfaces was significantly higher than to untreated surfaces at 0, 1, 3 and 7 h ($p < 0.05$ Student's t-test) at 12 h ($p < 0.1$) but not at 24 h ($p > 0.1$).

Figure 3.31 Adhesion of *L. monocytogenes* CRA 433 to plasma-glow discharged dental wax surfaces over a 24 h incubation period at 10°C.



Surface types.

■ = Untreated dental wax.

▲ = Plasma-glow discharged dental wax.

------ M.D.L (minimum detection level of photometric assay = 5.07 x 10^6 cells cm^{-2}).

Data represent adhesion levels over a 24 h incubation period at 10°C for *L. monocytogenes* cells suspended in 40 mM $MgCl_2.6H_2O$ to two surface types.

Values below the M.D.L were calculated using SEM adhesion analysis, total bacterial counts were made in ten random regions over the surface of each of three replicate samples ± standard errors of the mean (bars).

Values above the M.D.L were calculated using photometric adhesion analysis for ten replicate samples ± standard errors of the mean (bars).

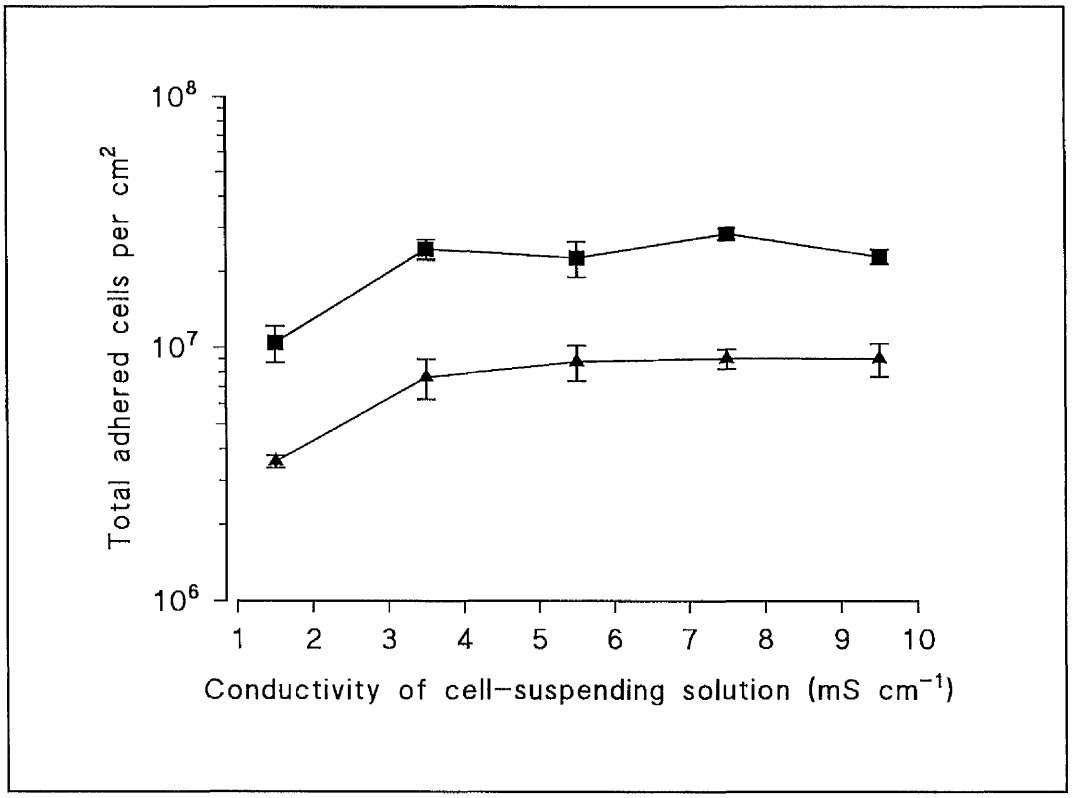
For PGD wax surfaces, adhesion increased at a logarithmic rate over the first 3 h of incubation and then began to plateau to a maximum level at 7 h. Adhesion at 7, 12, and 24 h was not significantly different ($p > 0.1$, Multiple Range test). The rate of adhesion to untreated wax surfaces was lower than to PGD surfaces; adhesion did not reach a maximum level until 24 h of incubation for untreated surfaces. At 24 h, adhesion to the two surfaces was not significantly different ($p > 0.1$ Student's t-test).

3.6.7 Effect of cell-suspending medium conductivity on the adhesion of *L. monocytogenes* CRA 433 to dental wax at 24 h.

With reference to the calibration graph of molarity against conductivity (illustrated in Appendix B), solutions of $MgCl_2.6H_20$ and NaCl were prepared at conductivities of 1.5, 3.5, 7.5 and 9.5 mS cm^{-1} in deionized water. For $MgCl_2.6H_20$, these were equivalent to molar concentrations of 6.67, 18.17, 29.58, 40.99 and 52.40 mM respectively. Similarly, for NaCl these conductivities were equivalent to 13.56, 32.55, 51.53, 70.52 and 89.50 mM solutions. One batch of *L. monocytogenes* CRA 433 was then resuspended in each of these solutions and adhesion to dental wax at 24 h measured using photometric and SEM adhesion analysis where appropriate.

Adhesion levels for all cell-suspending solutions are illustrated in Fig. 3.32. At all conductivities the adhesion for cells suspended in $MgCl_2.6H_2O$ were significantly higher ($p < 0.05$) than for NaCl. For $MgCl_2.6H_2O$ suspended cells, mean adhesion levels increased significantly from 1.04×10^7 cells cm^{-2} (S.E. = 1.71×10^6) at 1.5 mS cm^{-1} (6.76 mM) to 2.46×10^7 cells cm^{-2} (S.E. = 2.24×10^6) at 3.5 mS cm^{-1} (18.17 mM). Adhesion did not increase significantly thereafter ($p > 0.1$). For NaCl, adhesion also increased significantly ($p < 0.05$) from a conductivity of 1.5 mS cm^{-1} (13.56 mM) to a maximum level by a conductivity of 3.5 mS cm^{-1} (32.55 mM).

Figure 3.32 Adhesion of *L. monocytogenes* to dental wax at 24 h when suspended in $MgCl_2.6H_2O$ and NaCl of equal conductivity.



Cell-suspending media.

■ = $MgCl_2.6H_2O$.

▲ = NaCl.

Data represents adhesion levels for *L. monocytogenes* cells in two cell-suspending media having conductivities of 1.5, 3.5, 7.5 and 9.5 mS cm^{-1}.

Values below 5.07 x 10^6 cells cm^{-2} were calculated using SEM adhesion analysis, total bacterial counts were made in 10 random regions over the surface of each of three replicate samples ± standard errors of the mean (bars).

Values above 5.07 x 10^6 cells cm^{-2} were calculated using photometric adhesion analysis for 10 replicate samples ± standard errors of the mean (bars).

3.6.8 Addition of increasing molar solutions of NaCl to a low molar solution of $MgCl_2.6H_2O$ and its effect on adhesion of *L. monocytogenes* CRA 433 to dental wax at 24 h.

In the previous section, adhesion for *L. monocytogenes* cells suspended in $MgCl_2.6H_2O$ increased from 1.04 x 10^7 cells cm^{-2} at 1.5 mS cm^{-1} (6.76 mM) to 2.46 x 10^7 cells cm^{-2} at 3.5 mS cm^{-1} (18.17 mM). The question arose, whether this increase in adhesion levels from 1.5 to 3.5 mS cm^{-1} would also be observed if the conductivity of a low molar $MgCl_2.6H_2O$ solution was raised from 1.5 to 3.5 mS cm^{-1} by the addition of solutions of NaCl of increasing molarity. The addition of 30 mM NaCl to a 6.67 mM solution of $MgCl_2.6H_2O$ increased the total conductivity of the solution from 1.5 to 4.73 mS cm^{-1}.

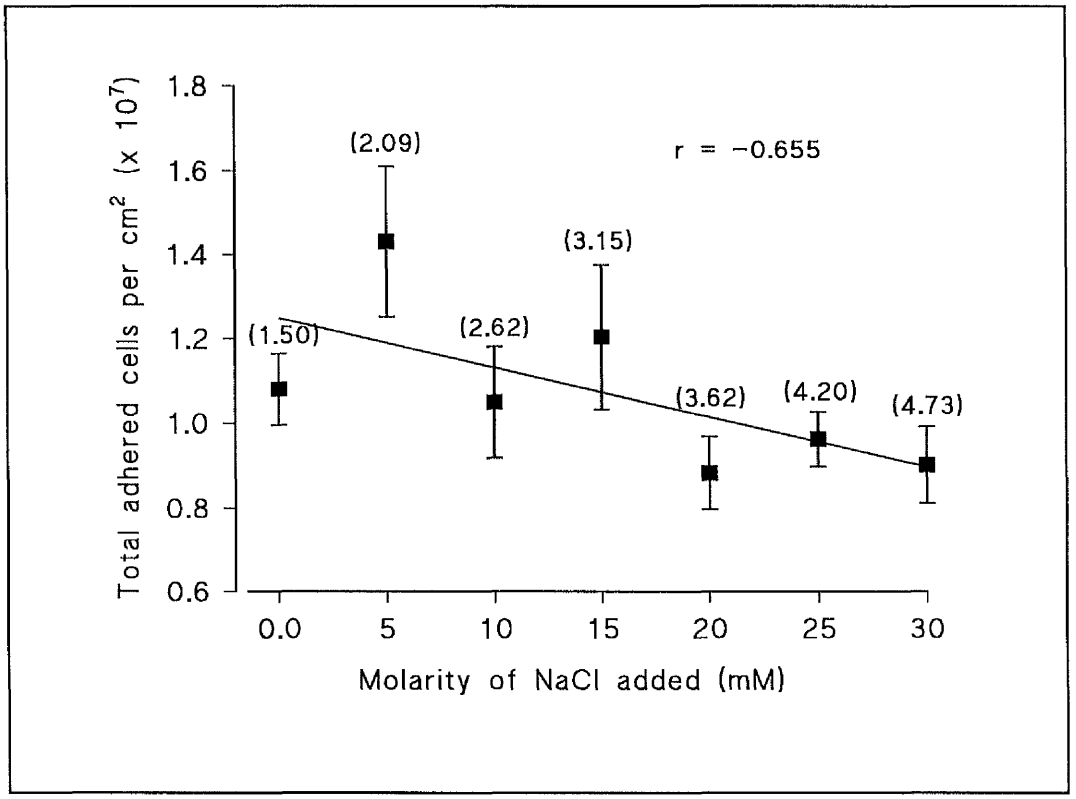
Figure 3.33 illustrates adhesion levels for *L. monocytogenes* cells suspended in the control $MgCl_2.6H_20$ solution (6.76 mM, 1.5 mS cm^{-1}) and combined solutions of 6.76 mM $MgCl_2.6H_20$ and NaCl of increasing molarity. No significant increase ($p > 0.1$, ANOVA) in adhesion levels from the control level was observed. Surprisingly, the general trend in adhesion levels seemed to decrease slightly with the addition of solutions of NaCl of increasing molarity. The decrease between 0 mM NaCl and 30 mM NaCl was, however, not significant ($p > 0.1$ Student's t-test). Adhesion could not therefore be increased to saturation levels observed for $MgCl_2.6H_2O$ simply by increasing the conductivity of the suspending medium. This suggests an additional role in adhesion for divalent cations compared to monovalent cations.

3.6.9 Effect of dimethyl sulfoxide on the adhesion of *L. monocytogenes* CRA 433 to dental wax at 24 h.

L. monocytogenes CRA 433 was resuspended in 0, 3, 6, 9, 12, 15, and 18% (v/v) solutions of dimethyl sulfoxide (DMSO) in 18.17 mM $MgCl_2.6H_2O$ a total cell concentration of 8.0 x 10^8 cells ml^{-1}. Dental wax was inoculated and adhesion measured following a 24 h incubation period at 10°C using photometric adhesion analysis.

Figure 3.34 illustrates adhesion levels over the range of DMSO concentrations. The addition of 3% DMSO to the cell-suspending medium reduced adhesion by

Figure 3.33 Effect of the addition of NaCl of increasing molarity to a low molarity solution of $MgCl_2.6H_2O$ on the adhesion of *L. monocytogenes* to dental wax.



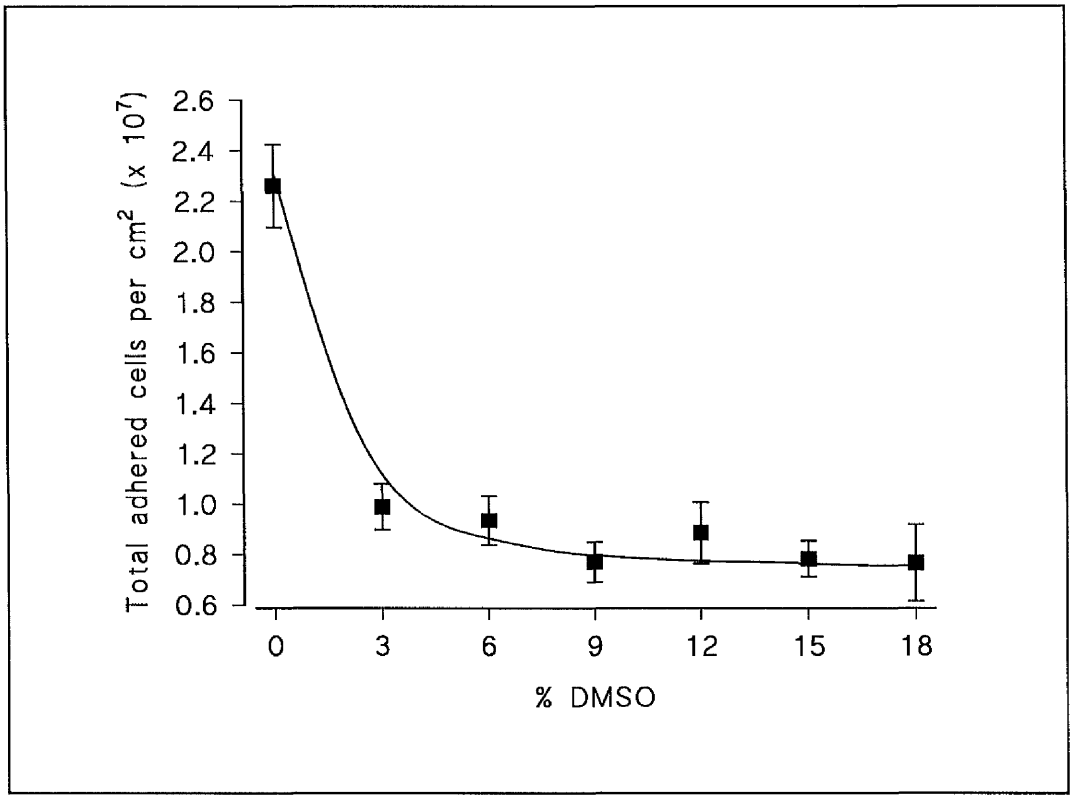
Data represent adhesion levels for *L. monocytogenes* cells suspended in a 6.76 mM $MgCl_2.6H_20$ solution with increasing molar solutions of NaCl.

Figures in parentheses indicate the total conductivity of the cell-suspending solution.

———— Linear regression correlation coefficient, r = -0.655.

Values were calculated using photometric adhesion analysis for ten replicate samples ± standard errors of the mean (bars).

Figure 3.34 Effect of dimethyl sulfoxide on the adhesion of *L. monocytogenes* CRA 433 to dental wax at 24 h.



Data represent adhesion levels for *L. monocytogenes* cells suspended in 18.17 mM $MgCl_2.6H_2O$ with the addition of 0, 3, 6, 9, 12, 15, and 18% (v/v) dimethyl sulfoxide (DMSO).

Values were calculated using photometric adhesion analysis for ten replicate samples ± standard errors of the mean (bars).

approximately 60% from the control level. This decrease was significant ($p < 0.05$). Adhesion levels did not decrease significantly above a 3% concentration of DMSO ($p > 0.1$, Multiple Range test).

3.6.10 Effect of eight biological surface-active agents on inhibition of *L. monocytogenes* adhesion to dental wax, and on adherent cells.

Eight biological surface-active agents (surfactants) (described in Section 2.12.4.ii) were selected. These were of four broad categories, namely;

i) Non-ionic:- Sorbitan monolaurate (**Span 20**), 10 Cetyl ether (**Brij 56**), Polyoxyethylenesorbitan- monolaurate (**Tween 20**) and monooleate (**Tween 80**).

ii) Anionic:- Sodium dodecyl sulphate (**SDS**).

iii) Cationic:- Benzethonium chloride (**BC**) and Cetyldimethylammonium bromide (**CB**).

iv) Zwitterionic:- [(3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate) (**CHAPS**).

The effect of these eight surfactants on the adhesion of *L. monocytogenes* to dental wax (Section 3.6.10.i) and effect on adherent cells (Section 3.6.10.ii) were investigated.

3.6.10.i Effect of biological surfactants on the adhesion of *L. monocytogenes* to dental wax.

The eight biological surfactants, detailed above, were prepared at concentrations of 50 and 100 ppm (v/v) in 18.17 mM $MgCl_2.6H_2O$. One batch of *L. monocytogenes* cells was divided into 17 equal volumes and each resuspended to a total cell concentration of 8.0×10^8 cells ml^{-1} in either one of the surfactants. Inoculum for control samples were resuspended in a 18.78 mM solution of $MgCl_2.6H_2O$. Surfaces were washed with ten dips in distilled water at 24 h and adhesion measured using photometric adhesion analysis.

An adhesion level for the control treatment of 2.1×10^7 cells cm^{-2} was recorded. As previously determined the minimum detection level (M.D.L) of the photometric adhesion assay was 5.07×10^6 cells cm^{-2}. Therefore, a reduction in adhesion from the

control to the MDL represents approximately a 75% decrease in adhesion. All surfactants reduced adhesion by this 75% when at a 100 ppm concentration. At the 50 ppm concentration, two compounds, namely, Brij 56 and CHAPS also reduced adhesion levels by at least 75%. Reductions for the remaining six compounds at the 50 ppm concentration were 70% (Span 20), 68% (Tween 80), 66% (Tween 20), 61% (SDS), 58% (CB) and 40% (BC). All of these results represented significant reductions from the control treatment ($p < 0.05$).

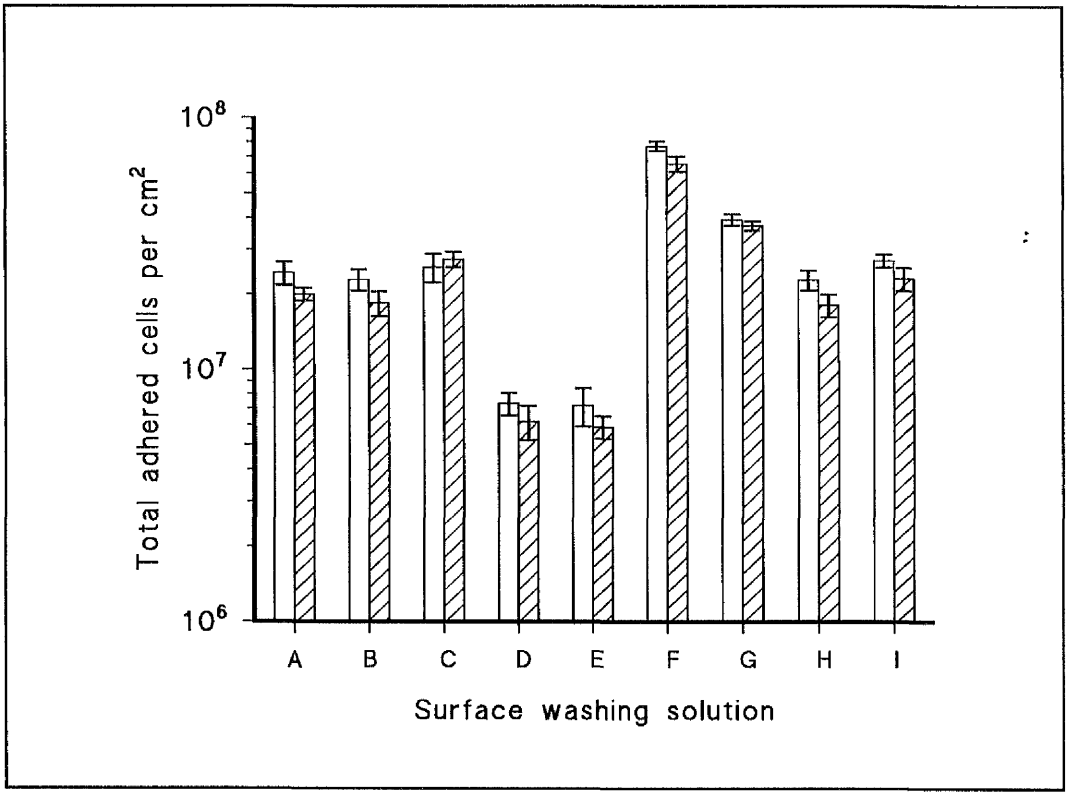
3.6.10.ii Effect of surfactants on adherent *L. monocytogenes* cells on dental wax.

3.6.10.ii.a Screening of surfactants for relative activity.

L. monocytogenes cells suspended in 18.17 mM $MgCl_2.6H_2O$ were allowed to adhere to dental wax over a 24 h incubation period at 10°C. The eight biological surfactants, detailed previously, were prepared at a very high 10000 ppm (1% v/v) concentration in sterile distilled water. Each surfactant was used to wash inoculated dental wax surfaces at 24 h with either eight or 18 dips plus two dips in distilled water (Section 2.12.4.ii). Control surfaces were washed with 10 or 20 dips in distilled water. Adhesion was measured using photometric adhesion analysis.

Figure 3.35 illustrates adhesion levels for the 16 surfactant and two control washing treatments. For each treatment adhesion was not significantly different ($p > 0.1$) between 10 and 20 dips. Two surfactants, namely, Brij 56, and SDS reduced adhesion significantly ($p < 0.05$) by 75% from the control treatments. For the two cationic surfactants, namely, BC and CB, adhesion levels were approximately 2.8 and 1.6 times higher than the control treatments. This indicates that these two surfactants, when added to distilled water, actually reduced the efficiency of distilled water in removing adhering cells. This would probably have occurred through a reduction in the washing shear forces which were produced over the dental wax surfaces in comparison to distilled water. The remaining four surfactants did not reduce adhesion levels significantly from control treatments ($p > 0.1$).

Figure 3.35 Effect of eight surfactants on adhered *L. monocytogenes* CRA 433 on dental wax surfaces using two washing protocols.



Washing solutions.
Non-ionic surfactants: A = Span 20, B = Tween 80, C = Tween 20, D = Brij 56.
Anionic surfactant: E = Sodium dodecyl sulphate (SDS).
Cationic surfactants: F = Benzethonium chloride (BC), G = Cetyldimethylammonium bromide (CB).
Zwitterionic surfactant: H = CHAPS
Control: I = distilled water.

Washing protocols.

□ = total of 10 dips.

▨ = total of 20 dips.

Data represents adhesion levels to dental wax at 24 h for surfaces washed with either of nine washing solutions using two procedures.

Values were calculated using photometric adhesion analysis for ten replicate samples ± standard errors of the mean (bars).

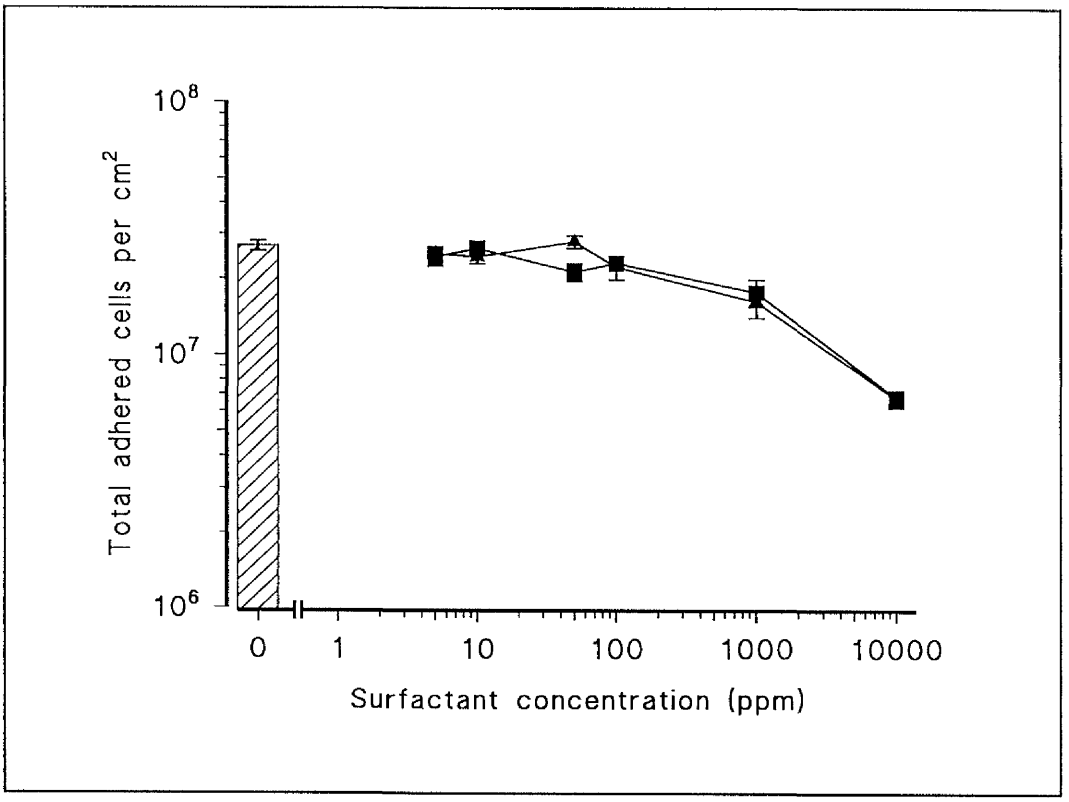
3.6.10.ii.b Effective concentration of Brij 56 and SDS for the removal of *L. monocytogenes* from dental wax.

The previous experiment demonstrated that at a concentration of 10000 ppm the surfactants Brij 56 and SDS could be used to reduce adhesion levels by approximately 75% from control levels. This surfactant concentration was very high and impractical for industrial use. The effective concentration (concentration at which adhesion was significantly reduced from the control treatment) of these two surfactants was, therefore, determined in this experiment using photometric adhesion analysis.

Adhering cells on dental wax were prepared as described previously. Brij 56 and SDS were prepared at concentrations of 10000, 1000, 100, 50, 10, and 5 ppm in sterile distilled water and each used to wash (eight dips in surfactant + two dips in water) inoculated surfaces following the 24 h incubation period. Control surfaces were washed with ten dips in distilled water.

Figure 3.36 illustrates adhesion levels for all washing treatments. At each concentration, adhesion levels between SDS and Brij 56 were not significantly different ($p > 0.05$). Adhesion was not reduced significantly from the control treatment at or below a 100 ppm concentration of either surfactant ($p > 0.1$, Multiple Range test). Significant decreases ($p < 0.05$, Multiple Range test) for both surfactants were observed from 100 to 1000 ppm and from 1000 to 10000 ppm concentrations. At 1000 and 10000 ppm concentrations adhesion was reduced by 40% and 75% from the control level respectively. The effective concentration of both surfactants was therefore above 100 ppm but below 1000 ppm.

Figure 3.36 Effect on adhesion of *L. monocytogenes* CRA 433 to dental wax of two surfactants.



Washing solutions.

▨ = Control: distilled water.

■ = Non-ionic surfactant: Brij 56.

▲ = Anionic surfactant: Sodium dodecyl sulphate (SDS).

Data represent adhesion levels to dental wax at 24 h for surfaces washed with either of two surfactants at a range of concentrations. All surfaces were washed with a total of 10 dips.

Values were calculated using photometric adhesion analysis for ten replicate samples ± standard errors of the mean (bars).

Chapter Four

Discussion

4.1 Bacterial adhesion assays.

In order to determine the factors affecting adhesion to cabbage leaf, lettuce leaf and model surfaces, adhesion assays were developed. Adhesion was quantified using three techniques, SEM adhesion analysis, microbiological counts and photometric adhesion analysis. The advantages and disadvantages of these techniques are described below.

The microbiological count assay was effective in quantifying both adhering and planktonic cells on lettuce and cabbage leaf surfaces. Maximum percentage adhesion levels for lettuce were statistically reproducible between experiments (Sections 3.2.1 & 3.2.2), however, variability between replicate samples was relatively high when compared to planktonic cells. Several factors may have contributed to this variability. For example, the localisation of adhering *L. monocytogenes* to the leaf cell margins of lettuce tissue (observed using SEM analysis, Section 3.1.2.ii.a) may have resulted in bacterial aggregates being recovered rather than single cells. Matthysse (1995) noted that bacterial aggregates may cause problems with the repetition of experiments because a bacterial aggregate will give the same adhesion level as a single cell when plated onto agar. Initial experiments (not presented) illustrated that such variability could be reduced significantly by macerating each sample thoroughly before plating onto agar, and that a pestle and mortar was more effective than a stomacher for this purpose.

Additional variability in microbiological adhesion assays may originate from inherent differences in the nature of the leaf surfaces themselves. For example, a leaf disc selected from an internal leaf may possess more surface irregularities, folds or even cell margins than an external leaf and consequently may provide more sites for bacterial adhesion. This experimental error can, however, be reduced by selecting leaf discs from the same location on each leaf surface. Experimental error may also occur when low adhesion levels are measured in the presence of high numbers of planktonic cells. This

situation was generally more apparent for lettuce tissue than cabbage tissue as indicated by the low 'true' percentage adhesion levels for lettuce (1.93 % for 1/4 strength Ringer's solution at 1 d, Section 3.2.1) and high levels for cabbage (20.44 % for 1/4 strength Ringer's solution at 1 d, Section 3.2.4.i). Sequential washing of leaf discs reduced the probability of planktonic cells persisting in the final leaf macerate and was, therefore, used to reduce this experimental error.

Total bacterial counts of adhering *L. monocytogenes* using SEM analysis provided a very accurate and reproducible technique for quantifying total cells on both leaf and model surfaces. A comparison between adhesion levels to dental wax at 24 h for four separate experiments (each experiment using a different batches of cells) showed that variation between experiments was very small (Section 3.5.4). Additionally, this technique provides visual images illustrating the distribution of adherent cells. Matthysse (1995) recently reviewed several methods currently available for observing and quantifying adherent microorganisms on exterior leaf surfaces, roots and root cap cells, the interior of plant, wounded tissue and tissue culture cells. Matthysse (1995) noted that any study which examines the adhesion of bacteria to plant tissue should use both quantification and imaging techniques simultaneously because the meaning of adhesion levels can only be assessed if the spatial distribution of the bacteria is also known.

One of the main advantages of SEM adhesion analysis over the microbiological count technique was that it allowed an increased number of sampling points to be examined over a 24 h period. Initial sample preparation was reduced because procedures such as maceration, cell dilution and agar plating were not necessary. Additionally, fewer replicate samples were required to achieve the same degree of accuracy observed with the microbiological technique. The technique did have certain disadvantages, for example, adherent cells on lettuce leaf tissue could not be quantified due to their localisation in cell margins (Section 3.1.2.ii.a). In addition, although initial sample preparation was minimal, subsequent manual quantification of cells was extremely labour intensive and required the use of expensive analytical equipment, i.e. an SEM. The technique could be modified to use image analysis and image handling techniques

(Busscher & van der Mei, 1995; Kim & Frank, 1995) thus removing the need to count adhered cells manually. Furthermore, the cost of the assay could be reduced if light microscopy was used instead of SEM. This was achieved successfully by Johnson (1995) who used light microscopy to quantify adhering cells on therminox coverslip surfaces. This technique was, however, limited to applications where the surface was transparent and adhesion levels low; clumping of adherent cells on the surface made it difficult to distinguish and count individual cells (Johnson, personal communication).

Recently, immunofluorescent labelling has been combined with conventional light microscopy and confocal laser scanning microscopy to provide *in situ* studies of species distribution and diversity in biofilms. However, quantification of cells using these techniques is still difficult due to problems of distinguishing individual bacteria (Amann *et al.*, 1992; Rogers & Keevil, 1992; Gorman, Mawhinney & Adair, 1993). Helke, Somers, & Wong (1993) used epifluorescence microscopy coupled with acridine orange staining to quantify *L. monocytogenes* and *Salmonella typhimurium* cells on Buna-N rubber and stainless steel surfaces. This technique did, however, still required manual counting of cells. Mosteller & Bishop (1993) compared impedance microbiology and epifluorescent filter techniques for quantifying *Pseudomonas fluorescens*, *Yersinia enterolytica* and *L. monocytogenes* adhesion to rubber and teflon surfaces. They found that impedance microbiology was the best method of enumeration, since it allowed the estimation of both reversible and irreversible attachment.

The photometric adhesion assay using crystal-violet staining of adherent cells provided a simple and rapid method for quantifying high numbers of adherent bacteria on dental wax surfaces. The main limitation of this technique was its relatively high minimum detection level. The assay could only detect bacterial populations in excess of approximately 5.0×10^6 cells cm^{-2} (see Section 3.6.1.iii) compared to the SEM adhesion assay which could quantify bacterial populations as low as 1136 cells cm^{-2} (see Section 2.11.2.ii). Nevertheless, since bacterial counts made by SEM and photometric analysis correlated well at high adhesion levels (see Section 3.6.1.iii) it was possible to combine both of these techniques in order to quantify low and high adhesion levels within one experiment.

Radiolabelling of bacterial cells prior to inoculation may provide an alternative technique for quantifying adherent *L. monocytogenes* cells in future studies. Radiolabelling may be used to quantify spatially distributed or clumped bacteria on a substratum van der Mei *et al.* (1993). Radiolabelling has been used successfully to quantify adherent staphylococci on polyurethane catheter surfaces (John *et al.*, 1995) and adherent *Pseudomonas fluorescens* on polyvinylchloride surfaces (Jones *et al.*, 1996). It should be noted, however, that batch variability was encountered in these studies; such variability may cause significant problems if used to measure adhesion on leaves where inherent variability in the leaf surface may lead to further experimental error. Matthysse (1995) noted that problems of quenching caused by the leaf tissue were often encountered when measuring adhesion of bacteria to leaf tissue using radiolabelling. These problems are exaggerated when the plant tissue is coloured, such as leaf discs of carrot. Consequently the technique may prove to be effective only when adhesion levels are relatively high. In spite of these drawbacks, the simplicity of radiolabelling as a technique for quantifying adherent cells does warrant further investigation for use in *L. monocytogenes* adhesion studies.

Adhesion of *L. monocytogenes* viable cells to stainless steel surfaces has also been measured using bacterial bioluminescence (Walker *et al.*, 1993). Such an approach may be used in conjunction with radiolabelling techniques to provide alternative methods to compare total and viable *L. monocytogenes* populations on model and leaf surfaces in future studies.

4.1.1 Effect of washing procedures on the measurement of adhesion levels.

Because the initial adhesion of bacteria is usually found to be reversible (Marshall *et al.*, 1971; Fletcher, 1977; Absolom *et al.*, 1983; Busscher *et al.*, 1984a; Puepke & Benny, 1984; van Pelt *et al.*, 1985; van Loosdrecht *et al.*, 1987a; van Loosdrecht *et al.*, 1989) and thus relatively weak, surface shear forces, hence washing procedures, may have a significant influence on the level of bacterial adhesion measured (van Haecht *et al.*, 1984). Powell & Slater (1982) found that for a surface shear, the force parallel rather than the force perpendicular to the surface governs detachment.

This parallel force exerted on the cell is proportional to r^2 (where r = the radius of the particle). Therefore, shear forces are more effective in detaching large particles such as bacteria, than smaller ones.

Busscher & van der Mei (1995) noted that washing procedures designed to remove loosely adhered cells may vary considerably in the shear forces which they produce and consequently the numbers of microorganisms they remove. They demonstrated that the removal forces involved in passing an adherent organism through a liquid-air interface ('dipping') are approximately 10^{-7} N, whereas, for an average flow of aqueous fluid in 'slight rinsing' a removal force of about 10^{-9}-10^{-10} N was estimated. It is, therefore, extremely important to standardise a washing procedure as much as possible in adhesion assays as variability will directly determine the number of adherent bacteria measured. The high variability usually found in adhesion experiments, i.e., 15-25% (Hoght *et al.*, 1983; van Pelt *et al.*, 1985; Kjuepfel, 1986; van Loosdrecht *et al.*, 1989), is probably partly caused by difficulties in standardizing the washing procedure (van Loosdrecht *et al.*, 1989).

Numerous studies have detailed adhesion levels for a host of bacteria to a range of different surfaces, however, a quantitative comparison between these results is often a difficult exercise as many of these studies use different washing procedures or do not even state the washing procedure used. This emphasises the need to thoroughly describe any washing procedure used in an adhesion assays.

In this study a standard washing procedure of 10 dips in distilled water was used to remove loosely (or reversibly) adhered cells. Cells which were quantified, either using SEM analysis, microbiological counts or photometric analysis were classified as being irreversibly adhered to these surfaces if they were not removed by washing.

Repetition of the standard washing procedure described above (i.e. using 20 dips in distilled water instead of 10) did not decrease the adhesion levels to dental wax surfaces significantly (Section 3.6.10.ii.a). This indicates that the magnitude of the shear force may determine the number of bacteria which become irreversibly adhered and not the number of times the shear force is applied. Experiments which compared the efficacy of a range of biological surfactants and distilled water on the removal of

adherent *L. monocytogenes* cells from dental wax surfaces (Section 3.6.10.ii.a) showed that two surfactants, namely, benzethonium chloride (BC) and cetyldimethylammonium bromide (CB) decreased the number of reversibly adhered cells which were removed (i.e. adhesion levels increased significantly compared to controls). Addition of these surfactants to water may have decreased the shear forces which the water produced over the surface, therefore increasing adhesion levels.

4.2 Reversible vs irreversible adhesion.

Bacteria may adhere to surfaces via two mechanisms, namely, by generic physico-chemical forces (Absolom *et al.*, 1983; Hoght *et al.*, 1983; Busscher *et al.*, 1984a, Busscher *et al.*, 1992; Busscher *et al.*, 1992; Olivera *et al.*, 1992), which are usually reversible, or by using surface structures or polymers of the cell such as pili, fimbriae or other appendages to form irreversible bonds (Costerton, 1974; Dazzo, 1984; Shea & Williamson, 1990). When approaching a surface from a long distance, an organism will first be exposed to physico-chemical forces (as described by the DLVO theory, see Section 1.15.3) before surface structures become operative. This successive interplay of forces (long-range generic followed by short-range specific) has been suggested by adhesion experiments with *Agrobacterium* and plant tissue (Matthysse *et al.*, 1981; Puepke & Benny, 1984). In situations where adhesion is not directly expected to be a specific process, for instance in soils and biofouling, long-range interactions are always responsible for the first step in the adhesion of the bacteria and will determine whether a firm irreversible adhesion occurs and ultimately the number of bacteria which form irreversible bonds.

4.2.1 Time taken to form irreversible bonds.

Although several reports have shown that *Listeria* may be isolated from vegetation and salad vegetables, indicating their ability to adhere to these surfaces, to my knowledge, only Wilson (1994) has studied the adhesion of *L. monocytogenes* to leaf surfaces. Wilson (1994) used scanning electron microscopy and microbiological counts to quantify adhering *L. monocytogenes* on adaxial leaf surfaces of Dutch White cabbage

following a 24 h incubation period at 10°C (cells were suspended in 1/4 strength Ringers solution). Using microbiological counts she found adhesion to increase significantly from 24 h to maximum levels by 3 d following incubation. In this present study *L. monocytogenes* were capable of adhering irreversibly after only short contact times (surfaces were washed immediately after inoculation) indicating some instantaneous irreversible adhesion, however, adhesion levels did increase significantly to maximum levels by 24 h (Section 3.2.4.i) indicating that additional time was required for maximum irreversible adhesion to occur. Similarly, maximum adhesion of *L. monocytogenes* to Iceberg lettuce tissue (Sections 3.2.1 & 3.2.2) and model dental wax surfaces (Section 3.5.1) also occurred within 24 h. Further experiments are required to determine the actual time required (between 0 and 24 h) for adhesion to reach maximum levels on cabbage and lettuce tissue. Radiolabelling may provide a more accurate and less labour intensive technique than microbiological counts for this purpose.

The time required for irreversible adhesion to occur may vary considerably depending on bacterial species and the substratum under study. Helke *et al.* (1993) showed that *L. monocytogenes* could adhere irreversibly to Buna-N rubber and stainless steel surface after contact times of 1 h at room temperature, however, the time required to reach maximum adhesion levels was not determined. Oh & Marshall (1996) showed that the numbers of *L. monocytogenes* adhering to stainless steel surfaces immersed in TSB at 25°C increased slowly between 1 and 7 d reaching a maximum level of 2.3 x 10^6 cfu cm^{-2} by 7 d. Krysinsky *et al.* (1992) tested adhesion of a three strain cocktail of *L. monocytogenes* on stainless steel, polyester and polyester baked with polyurethane surfaces. They found that the type of surface had little effect on the levels of attachment, adhesion reached maximum levels by 24 h. Jones *et al.* (1996) showed that maximum adhesion of *Pseudomonas fluorescens* to polyvinylchloride was reached by 6 h, however, other studies have reported maximum adhesion occurring after less than 5 min for a *Pseudomonas* strain adhering to hydrophobic polystyrene (van Loosdrecht *et al.*, 1989).

The long incubation period required to reach maximum adhesion levels on dental wax surfaces does suggest, however, that firm adhesion may require the production of

extracellular material by the bacteria once reversibly adhered to the surface. Fletcher & Floodgate (1973) recognized two alcian blue- and ruthenium red-reactive extracellular polymers produced by a marine pseudomonad attached to surfaces. One was a compact layer on the outer surface of the cell wall, which the authors suggested was involved in the adhesion process, and the second was a looser fibrous polysaccharide, which may have been produced by the bacterium after attachment. Similarly, Costerton *et al.* (1981) described the production of additional extracellular polymeric material after adhesion of a *Pseudomonas* spp., followed by the replication of the bacterium within the polymer matrix to form microcolonies. Polysaccharide production by *L. monocytogenes* will be discussed in later sections (see Section 4.3.1).

In addition to the production of extracellular material, several other factors will also affect the time taken for maximum adhesion levels to be reached. For example, transport mechanisms of the bacteria to the surface will determine the time taken for all adhesion sites on a surface to become saturated. If bacteria deposit onto a surface rapidly for example, then all adhesion sites will be filled quickly (assuming cell density to be sufficiently high to saturate the surface) and the time taken for the initial reversible adhesion to occur will be short. Results in this study have indicated that cell deposition onto dental wax surfaces can significantly increase the adhesion levels reached within a 24 h incubation period (Section 3.6.4). Consequently, a model system where the substratum was immersed in a suspension of bacterial cells may lead to lower adhesion levels being observed at 24 h than those in this study.

The ability of bacteria to approach a surface by their own movement e.g. using flagella, will also increase the probability of the bacteria coming into direct contact with the surface within a given time period and thus forming reversible and irreversible bonds. Motile *Pseudomonas fluorescens* cells have been found to adhere 4 times faster than non-motile strains (Korber *et al.*, 1994). The importance of cell motility in adhesion was not studied in this project and merits further investigation (see Section 4.3.1).

Although it is generally accepted that a certain time period is required for a bacterium which is reversibly adhered to a surface to become irreversibly adhered,

several factors may determine whether a firm irreversible adhesion will actually occur or not.

4.3 Factors affecting irreversible adhesion.

4.3.1 Presence of cell surface appendages on *Listeria* species detected by TEM and SEM.

Any fine polymeric component external to the cell envelope, such as extracellular polymers, pili, fimbriae and flagella may be responsible for the bridging of bacteria to solid surfaces and the formation of irreversible bonds. Studies of a wide variety of surfaces have confirmed the universality of polymer bridging as the means whereby bacteria bind irreversibly to surfaces (Costerton *et al.*, 1981).

Fimbriae and pili have been associated with the adhesion of Gram-negative bacteria such as *Pseudomonas syringae pv. syringae* to bean leaf surfaces (Romanshuk & Bamford, 1986) and *Klebsiella* and *Enterobacter* species to grass roots (Korhonen *et al.*, 1983; Haahtela *et al.*, 1989). Fimbriae of *Pseudomonas fluorescens* have been shown to mediate the adhesion of the bacteria to corn roots (Vesper, 1987). Additionally, adhesion to carrot cells has been shown to correlate with the possession of cellulose fibrils (Matthysse *et al.*, 1981) in *Agrobacterium tumefaciens*. As well as mediating the initial adhesion of *A. tumefaciens* to plant tissue, the cellulose fibrils of this microorganism have also been shown to entrap planktonic bacteria thus aiding their adhesion (Matthysse, 1995).

None of the *Listeria* species tested produced adhesive structures such as fimbriae, fibrils or pili using the culture conditions and media described (Section 3.3.1). Wilson (1994) also showed using negative staining that three *L. monocytogenes* isolates belonging to serogroups 3, 4b did not possess these surface appendages when grown overnight in BHI broth at 25°C. The visualisation of long, short and peritrichous fibrils on the control organism *Streptococcus sanguis* (CN3410) demonstrates the validity of the negative staining technique used for the detection of these structures, and the conclusion that the *Listeria* species investigated did not possess them.

The possibility still exists that *Listeria* cells do produce filamentous appendages

under growth regimes not studied in this dissertation. Furthermore, such appendages could potentially be produced or induced after bacterial contact with a substratum. Stemmer & Sequeira (1987) showed that the cell surface properties of an organism can change with growth conditions, factors such as temperature and whether the bacteria are growing in liquid or on solid culture may influence the production of surface structures such as fimbriae or pili. Furthermore, the presence of certain nutrients, such as glucose or analin) in the growth medium may also influence whether such structures are produced (Jann & Hoschutzky, 1990). Dawson, Humphrey & Marshall (1981) observed that the number of irreversibly adhered bacteria increased with exposure in a starvation regime for a marine *Vibrio* species. This increased adhesion was attributed to formation of fibrils when the cells were starved. Thus, further investigation using different growth conditions may yield different results. However, *L. monocytogenes* is generally considered to be non-fimbriate (Benedict, 1990).

Flagella were observed for all *Listeria* species examined and under each growth regime used. Some researchers have indicated that bacteria appear to be held firmly at a surface by the flagella (Meadows, 1971; Belas & Colwell, 1982). Flagella have been implicated as important in the adhesion of *Erwinia* strains to potato leaflets as indicated by the reduced adhesion levels for agar grown cells compared to their broth-grown counterparts (Wallace & Perombelon, 1993). The possibility does exist, therefore, that flagella are of primary importance in the adhesion of *Listeria* to surfaces. Flagella may provide both a propulsive force allowing the bacteria to approach the surface and in addition may act as a bridging polymer between the bacterium and substratum. *Listeria* is known to become non-motile at 37°C due to a reduction in flagellin production (Peel *et al.*, 1988). The importance of flagella in the adhesion of *L. monocytogenes* could, therefore, be investigated if a flagellin-negative mutant of the organism could be produced.

Polymeric materials extending between *Listeria* cells and the substratum were described by Mafu *et al.* (1990) in a SEM study investigating adhesion of *L. monocytogenes* to stainless steel, glass, polypropylene and rubber surfaces. Using SEM analysis, identical filamentous structures to those described by Mafu *et al.* (1990) were

also observed on cabbage tissue inoculated with *L. monocytogenes* cells (Section 3.1.2.i.a). Mafu *et al.* (1990) commented that these structures may play some role in the attachment of *Listeria* to surfaces, possibly by forming polymeric bridges and overcoming the repulsion barrier. He also noted, however, that they may have been produced as a result of condensation and chemical denaturation during specimen preparation. Matthysse (1995) supported this view and added that such artifacts may result if too rapid dehydration or inadequate fixation of the sample occurs. McCarthy (1992) also observed these structures associated with individual *L. monocytogenes* cells adhered to chitin surfaces and suggested that they were fimbriae. However, the length of some of the filamentous structures observed in this study suggests that they were not fimbriae, pili or fibrils. Additionally, the very straight appearance of these filamentous structures coupled with the fact that they were observed at elevated positions from the substratum, suggests that they were not flagella since when viewed under SEM, flagella are usually observed laying directly on the substratum in characteristic irregular patterns (Gunning *et al.*, 1996).

The possibility exists that the filamentous structures observed using SEM were dehydrated extracellular polysaccharide (EPS) which had been produced by the cells. Ronner & Wong (1993) showed using SEM analysis that EPS production by *L. monocytogenes* was higher on stainless steel surfaces than Buna-N rubber following 2 d incubation at room temperature (21-24°C), surfaces were incubated in Tryptose Phosphate broth for adhesion experiments. Herrald & Zottola (1988) showed, however, that although *L. monocytogenes* produced EPS at 21°C, it was not produced at either 10 or 35°C. Since in this study, both cell growth and surface incubation were performed at 10°C, it is unlikely that the cells produced EPS. Thus, if no EPS is produced at 10°C, but the cells still adhere, this suggests that EPS is of limited importance in the ability of *L. monocytogenes* to adhere irreversibly. This current study did not examine the production of EPS by *Listeria* species, therefore although no obvious EPS was visualised using SEM analysis, it is still possible that it was produced in small amounts and possibly only present in the junction between the bacterium and the substratum. Interestingly, Sasahara & Zottolla (1993) have shown that the attachment of *L.*

monocytogenes to glass surfaces was sparse when the bacteria were in pure culture at 23°C, however, when the cells were grown in mixed culture with *Pseudomonas fragi*, an extracellular polysaccharide (EPS) producing microorganism, attachment and microcolony formation by *L. monocytogenes* was enhanced. The authors suggested that *L. monocytogenes* may become trapped within the EPS matrix produced by *P. fragi* cells. Alternatively, *L. monocytogenes* may actually be attracted to the EPS matrix and adhere actively. Therefore, although the production of EPS does not seem necessary for *L. monocytogenes* adhesion to occur, the presence of EPS on a substratum may increase the adhesion levels observed, for example by forming a hydrophobic conditioning film.

Mesosomes were also observed for all *Listeria* species examined and the control organism *Streptococcus sanguis* (CN3410). Thorne & Baker (1972) found similar organelles primarily in species of various Gram-positive bacteria including species of *Bacillus*, *Lactobacillus*, *Staphylococcus* and *Streptococcus*. Ghosh & Murray (1967) described mesosomes in *L. monocytogenes* as being part of the plasma membrane which may be synthesised by the cell naturally or formed from a damaged plasma membrane. Using TEM examination of sectioned material, El-Kest & Marth (1992) reported the presence of mesosomes in *L. monocytogenes* Scott A cells following thawing of cells held in frozen storage for 7 d. They concluded that these mesosomes were formed due to a retraction of the cytoplasm and infolding of the plasma membrane either inside the cytoplasm or in the space between the cell membrane and cell wall. It is believed that their function is essentially to increase the cell membrane surface area, which in turn increases enzymatic content and promotes activities such as cell wall synthesis and respiration (El-Kest & Marth, 1992). However, these mesosomes have not been implicated as being of direct importance in bacterial adhesion although any structure which increases metabolic activity may affect adhesion indirectly, for example by increasing the rate of cellular motility or the production of extracellular polymers.

4.3.2 Effect of electrolytes on adhesion.

Several experiments in this study illustrated a very strong correlation between high adhesion levels of *L. monocytogenes* and the presence of electrolyte in the cell

suspending medium. Adhesion levels on cabbage (Sections 3.1.2.i.b & 3.2.4.i), lettuce (Sections 3.1.2.ii.b & 3.2.1) and dental wax surfaces (Sections 3.5.4 & 3.5.6.i) were increased when the cells were suspended in 1/4 strength Ringer's solution compared to deionised water.

Electrolytes can influence adhesion both by affecting long-range electrostatic forces and by influencing the expression of short range hydrophobic bonding. Each of these effects are described below.

4.3.2.i Significance of long range forces in adhesion of *L. monocytogenes* to leaf and model surfaces.

The significance of long-range electrostatic interactions has been investigated in several studies by determining the effect of electrolytes on attachment. The adhesion of bacteria to surfaces, for example, *Achromobacter* to glass (Marshall *et al.*, 1971), *Pseudomonas aeruginosa* to stainless steel (Denyer *et al.*, 1993) and *Vibrio alyinolytica* to hydroxyapatite (Gordon & Millero, 1984), has been increased by raising the electrolyte concentration in the suspending medium.

In contrast it has been suggested that in low ionic strength solutions the attractive forces between adhering *Streptococcus salivarius* is due to a polymer-bridging form of interaction between the extended fibrils of these cells. This is an example of positive cooperativity (see Section 4.4). In high ionic strength solutions these surface appendages collapse due to a screening of charge interactions, whereas, in low ionic strength solutions they probably extend far in the solution due to electrostatic repulsion (Sjollema *et al.*, 1990). Thus, in some cases where adhesion is mediated by polymer bridging, high ionic strength environments may result in a reduction in the number of cells which become irreversibly adhered.

Adhesion to dental wax surfaces was increased when the molarity of the cell suspending solution was increased (Section 3.5.5). This was observed using both monovalent and divalent cations. The effect of the electrolyte concentration on adhesion can be explained in terms of the overlap of diffuse double layers surrounding the bacterium and the substratum (Rutter & Vincent, 1984). The thickness of the diffuse

layer of counter charge surrounding a charged particle or bacterium is a function of ionic strength (van Loosdrecht *et al.*, 1989). According to the DLVO theory (see Section 1.15.3), in situations where both the bacterium and the substratum are of the same charge, the thickness of the diffuse layer decreases with increasing ionic strength of the medium, resulting in a decrease in the repulsive electrostatic interactions between the two surfaces. At intermediate electrolyte concentrations, the electrostatic repulsion forces will be reduced sufficiently to lead to the formation of a secondary attractive minimum where either weak reversible adhesion may occur, or if the minimum is very deep (i.e strong van der Walls attraction) irreversible adhesion may result. At high electrolyte concentrations electrostatic repulsion between the two surfaces is eliminated and the bacterium may approach the surface much closer and enter the primary minimum where strong short-range irreversible bonds may be formed.

Electrolytes may therefore allow the bacterium to approach the surface to a much shorter separation distance than would occur in a low electrolyte system. In low electrolyte solutions, adhesion levels may be low because few cells can approach the surface closely enough to form irreversible bonds.

Dickson & Siragusa (1994) determined that *L. monocytogenes* exhibited a net negative charge when suspended in Butterfield's phosphate buffer at pH 6.8. Mozes & Rouxhet (1987) indicated that the isoelectric point of most microorganisms is in the pH range 2 to 3.5. Thus, although the isoelectric point for *L. monocytogenes* has not to date been determined it can be assumed that for all adhesion experiments in this study the bacterial cells were negatively charged when inoculated onto the substratum as they were suspended in solutions at pH 6.8 or above. From this assumption it is highly probable that increased adhesion levels observed for *L. monocytogenes* on leaf and model surfaces were in some part the result of decreased electrostatic interactions between the two surfaces.

Irreversible adhesion in the presence of multi-valent counter ions is frequently observed (Nordin *et al.*, 1967; Marshall *et al.*, 1971; Turakhia *et al.*, 1983; Rouxhert *et al.*, 1984; Alieva *et al.*, 1987; van Loosdrecht *et al.* 1987b). In colloid chemistry the pronounced effect of the valency of the counter ions on electrostatic interactions can be

inferred from the concentration needed to destabilize a negatively charged AgI sol, i.e., 140 mM for $NaNO_3$, 2.4 mM for $Ca(NO_3)_2$, and 0.067 mM for $Al(NO_3)_3$ (Kruyt & Kjompe, 1942). Thus, a lower concentration of multi-valent compared to univalent cations is required to reduce the electrostatic repulsion between two particles with the same charge. As previously described, adhesion to lettuce and cabbage tissue and dental wax was higher in the presence of divalent cations than monovalent cations of equal concentration. This may be explained as being due to a greater double layer compression in divalent systems (Marshall *et al.*, 1971). Again an increased compression of the double layer results in more bacteria entering the primary minimum where strong irreversible adhesion may occur.

The selectivity with which plant pathogenic bacteria adhere to plant surfaces, has led to the general belief that adhesion between the plant and the bacterium itself is a specific process caused by a biochemical interaction. For example, the positive influence of Ca^{2+} on the adhesion of *Rhizobium leguminosarum* was explained by postulating the presence of a Ca^{2+} -dependent adhesion (Smit *et al.*, 1987). However, it has been suggested that it is more likely that Ca^{2+} decreases the electrostatic repulsion during the initial stage of adhesion and, therefore, the bacteria will adhere to plant surfaces the same way as they do to other surfaces (van Loosdrecht *et al.*, 1989).

As well as reducing electrostatic interactions, multi-valent cations may also promote adhesion through bridging interactions between the bacterial surface and the substratum (Zita & Hermansson, 1994). The presence of bridging interactions between *L. monocytogenes* and dental wax surfaces may explain why the addition of monovalent electrolytes to a low molar solution of cations did not result in an adhesion level as high as that observed for a divalent cation solution of equal conductivity (Section 3.6.7).

4.3.2.ii Significance of cell surface hydrophobicity in the adhesion of *L. monocytogenes* to leaf and model surfaces.

Bacteria exhibit variations in overall surface free energy of the cells, with some bacteria possessing relatively hydrophilic surfaces and others relatively hydrophobic surfaces (Mudd & Mudd, 1924; Magnusson *et al.*, 1977; Dahlbach *et al.*, 1981;

Busscher *et al.*, 1990; Doyle & Rosenberg, 1990). Hydrophobicity of *Listeria* was assessed using the BATH test of Rosenberg *et al.* (1984a). The proportion of *Listeria* cells which expressed a net hydrophobic nature was very low (3-4%) for each of five *Listeria* species when suspended in deionised water at 10°C (Section 3.3.2.i.a). Hydrophobicity for these isolates did not increase when the growth temperature was increased to 25°C. A high proportion of *Listeria* cells in each of these cell suspensions may, therefore, be characterised as being hydrophilic at both 10 and 25°C. Absolom *et al.* (1983) also characterised *L. monocytogenes* as being a relatively hydrophilic microorganism using water contact angle measurements on bacterial lawns. Similarly, Mafu *et al.* (1991) also classified the organism as hydrophilic using hydrophobic interaction chromatography (HIC) and observed that high ionic strengths favoured hydrophobic interactions between *L. monocytogenes* cells and amphiphilic gels.

The expression of net hydrophobicity for four *L. monocytogenes* isolates was found to increase significantly when the cells were suspended in 1/4 strength Ringer's solution compared to deionised water (Section 3.3.2.i.b). Additionally hydrophobicity for *L. monocytogenes* CRA 433 also increased significantly when cells were suspended in divalent cations compared to monovalent cations (Section 3.3.2.i.d).

Israelachvili (1985) explained the observation that hydrophobic interactions for bacterial cells are stronger in ionic solutions. He suggested that positively charged ions strongly associate with the water molecules surrounding the bacteria and that in high ionic concentrations the polymers on the bacterial surface become dehydrated, exposing hydrophobic domains on the cell surface. Consequently, as electrolyte concentration increases, the solvation barrier between the bacteria and the surface will decrease and thereby enhance the adhesion capacity to surfaces. In this study, hydrophobic hexadecane droplets were the surface to which the bacteria attached. The increased proportion of *L. monocytogenes* cells which expressed a net hydrophobicity was therefore the result of more cells expressing sufficient hydrophobic groups to partition from the water phase and adhere to the hexadecane.

Hypothetically the removal of interfacial water may be the main mechanism by which cell surface hydrophobicity influences bacterial adhesion. This dehydrating

capacity of bacteria occurs through hydrophobic groups associated with the cell surface or their surface appendages. With the removal of water between the bacterium and the substratum, the bacterium may approach the surface much closer and consequently the probability of forming irreversible adhesive bonds is increased (Busscher & Weerkamp, 1987).

Percentage adhesion levels to all surfaces studied indicated that a small proportion of inoculated cells became adhered, e.g. 3.69% of *L. monocytogenes* cells suspended in 1/4 strength Ringer's solution adhered to lettuce tissue (Section 3.2.3). The possibility, therefore, existed that only the cells expressing a net hydrophobic nature were capable of adhesion. This would explain the correlation observed between increasing hydrophobicity levels measured for different electrolytes (Section 3.3.2.i.d), and increasing adhesion levels. Attempts were made to remove hydrophobic cells from the inoculum so that adhesion to dental wax for the resultant 100 % hydrophilic population could be compared to a cell population containing approximately 90% hydrophilic and 10 % hydrophobic cells. Results showed that adhesion levels for both populations were not significantly different (Section 3.5.6.ii). Consequently, it is unlikely that the increased adhesion levels to leaf and model surfaces in the presence of electrolyte was directly correlated with a higher proportion of hydrophobic cells in the inoculum. In spite of these results, it is still possible that the expression of hydrophobicity conveys an advantage to the cells in adhesion; for example the hydrophobic cells in a population could potentially adhere to a surface before the hydrophilic cells.

A more defined approach for comparing the adhesion of hydrophobic and hydrophilic variants is to compare adhesion of 100% hydrophobic and 100% hydrophilic populations. Simon (1981) was able to isolate a hydrophobic *Aphanotheca halophytica* mutant from an essentially hydrophilic population of cells using the modified BATH test. He showed that variation in hydrophobicity was genetically determined and was, therefore, able to enrich the hydrophobic mutant to form two discrete populations of cells. In this present study attempts to enrich the hydrophobic *L. monocytogenes* cells from the predominantly hydrophilic population were unsuccessful (Section 3.3.2.ii).

This may indicate that the expression of hydrophobicity of *L. monocytogenes* cells is not genetically determined and that hydrophobicity may, therefore, be under phenotypic control.

Cell surface hydrophobicity has in the past been shown by many authors to be affected by growth conditions. For example, Kjelleberg & Hermanson (1984) reported an increase in the hydrophobicity with 4 of 7 marine isolates upon starvation, whilst in contrast, van Loosdrecht *et al.* (1987a) observed that some bacteria become more hydrophobic during exponential growth phases. Variations in hydrophobicity (measured by HIC) during growth of *Staphylococcus epidermidis* have been clearly correlated to variations in the adhesion of this microorganism (Gilbert *et al.*, 1991). The same author has shown that this does not apply for *Escherichia coli*. The degree of adhesion of this microorganism is inversely proportional to the negative electrical charge of its surface and bears no relation to its hydrophobicity. Results presented in this study showed that growth phase did not influence the hydrophobicity of *L. monocytogenes* when grown in TSB at 10°C (Section 3.3.2.i.b) but did influence adhesion (approximately 9 % of cells expressed a net hydrophobic nature when suspended in 1/4 strength Ringer's solution for mid-exponential, early- and late stationary). Whereas adhesion to dental wax was decreased for mid-exponential cells compared to early- and late stationary phase cells (Section 3.5.2). Therefore, adhesion could not explained in terms of altered cell surface hydrophobicity.

Maximum adhesion of *Bradyrhizobium japonicum* to soybean root segments has also been shown to be dependent on the bacterial growth phase of the cells (Smith & Wilson, 1991). Attachment of *B. japonicum* was increased 3- and 10-fold at stationary phase compared with log phase of growth respectively and was explained as a survival tactic in response to nutrient depletion in late growth phases. Starvation stress has often been reported to increase microbial cell attachment (Dawson *et al.*, 1981; Kefford, Humphrey & Marshall, 1986). The increased adhesion of *L. monocytogenes* to dental wax in later growth phases may, therefore, also be a survival tactic. However, Dickson & Frank (1993) found that starvation did not affect the attachment of *L. monocytogenes* to beef tissue.

Although no significant differences were found for the attachment of *L. monocytogenes* to lettuce tissue when grown in TSB and BHI broth (Section 3.2.2), Kim & Frank (1994) showed that adhesion of *L. monocytogenes* cells suspended in 20 mM phosphate buffer to stainless steel surfaces was 50 times higher for cells initially grown in the chemically defined media D10 than in TSB. The authors suggested that an increased hydrophobicity of cells grown in D10 would account for their increased adhesion to the hydrophilic substratum, however, cell surface hydrophobicity was not determined in this study. This is obviously a point of further investigation and may clarify the importance of *L. monocytogenes* cell surface hydrophobicity in its adhesion to surfaces.

L. monocytogenes F139 (1/2a) showed a lower net hydrophobicity in both deionised water and 1/4 strength Ringer's solution compared to three 4b *L. monocytogenes* isolates, namely, CRA 433, CRA 5246 and ATCC 23074 (Section 3.3.2.i.b). Interestingly, isolate F139 also showed lower adhesion to dental wax than the three 4b isolates when suspended in both 1/4 strength Ringer's solution (Section 3.5.6.i) and 40 mM $MgCl_2.6H_20$ (Section 3.6.3). Although the low adhesion of isolate F139 may have been directly related to its low hydrophobicity, no firm conclusion can be drawn from the data at this point. Further studies are required to measure the hydrophobicity of a number of other *L. monocytogenes* isolates so that a better correlation between cell surface hydrophobicity and adhesion can be made. Results presented in Section 3.6.3 did show that the low adhesion level of isolate F139 1/2a, compared to isolates CRA 433, CRA 5246 and ATCC 23074 (all serotype 4b), was not the result of it being of a different serotype to these isolates. This conclusion can be made because an additional *L. monocytogenes* 4b isolate, namely, CRA 1175, showed significantly lower adhesion to dental wax than *L. monocytogenes* F139.

Bacteria are frequently classified as hydrophobic or hydrophilic, however, this may confer the wrong impression. The BATH test measures only the net hydrophobicity of cells. Bacterial cells do, however, have both polar and non-polar molecules on their cell surface and depending on the amount and distribution of these groups they may express a certain degree of hydrophobicity. Additionally, the distribution of hydrophobic

sites on bacterial cells is not necessarily uniform and may result in a preferred orientation of certain bacteria at interfaces (Marshall & Cruickshank, 1973). Rosenberg & Kjelleberg (1986) used the terms hydrophobins and hydrophilins to describe surface components that confer hydrophobic and hydrophilic properties of the bacterial cell. They implicated fimbriae, fibrils, capsules, flagella and membrane proteins as possible hydrophobins and hydrophilins.

Colloidal gold labelling was used to identify hydrophobic sites on *L. monocytogenes* cells. Colloidal gold consists of a gold hydrophobic centre surrounded by a cloud of negatively charged groups, and therefore binds to hydrophobic and positively charged moieties (Handley *et al.*, 1990). If labelling is pH independent, this indicates that the gold is binding to hydrophobic groups only. Labelling of *L. monocytogenes* cells and their flagella when suspended in deionised water was extremely low for all pH levels studied (Section 3.3.2.iii.a). This indicates that under these conditions very few hydrophobic or positive groups are expressed either on the cell surface or on flagella. This correlates with results of the BATH test which indicated that most cells in suspension were hydrophilic (i.e expressed few hydrophobic groups).

When *L. monocytogenes* cells were suspended in 1/4 strength Ringer's solution they showed increases in both the mean level of gold labelling of the cell surface and the proportion of cells which became labelled (Section 3.3.2.iii.b). This was observed at all pH values tested. Increased labelling may result from increases in either the number of positive sites or hydrophobic groups on cell surface which were expressed in ionic solution compared to deionised water. The number of positive sites on the surface should increase as the pH of the medium decreases; the cell will carry a net positive charge only below its isoelectric point. However, it was observed that the mean level of labelling actually decreased as the pH of the cell suspension was lowered indicating that the cells had not reached their isoelectric point and still carried a net negative charge. This implies that the increase in colloidal gold binding in high ionic environments was due to the increased number of hydrophobic groups which were expressed on the cell surface. However, it is also possible that increased binding of gold to the cell surface was the result of the gold binding to divalent cations which had

themselves bound to the cell surface from the suspension. Results of this study are therefore inconclusive in the respect that it is not known whether increased binding of colloidal gold to *L. monocytogenes* in the presence of electrolytes is the result of an increased number of charged or hydrophobic groups on the cell surface. However, the results do indicate that *L. monocytogenes,* when in electrolyte solutions, does show an increased binding potential to negatively charged hydrophobic moieties than when in low ionic environments.

From an overview of cell surface hydrophobicity data presented in this study it is clear that the majority of *L. monocytogenes* cells express a net hydrophilic nature and a minority express a net hydrophobic nature. Therefore, *L. monocytogenes* can not strictly be defined as being either hydrophobic or hydrophilic. It is also clear that different levels of hydrophobicity existing in any one population of cells are dependant on the electrolyte concentration of the suspending medium. Consequently these cells may show different capacities to form reversible short range interactions with surfaces. Future research should concentrate on trying to separate hydrophobic from hydrophilic variants and to compare the adhesion capacities of these variants to different leaf and model surfaces.

4.3.3 Substratum surface properties.

Results presented in this study have shown that adhesion of *L. monocytogenes* to model surfaces correlated with both the substratum hydrophobicity (Section 3.4.3.ii.c) (measured using advancing water contact angles) and the substratum surface roughness (Section 3.4.3.ii.b) (defined using Ra measurements).

4.3.3.i Substratum hydrophobicity.

Many authors have found a good correlation between contact angle measurements and bacterial adhesion (Fletcher & Loeb, 1976, 1979; Absolom *et al.*, 1983; Pringle & Fletcher, 1983; Busscher *et al.*, 1984a; van Loosdrecht *et al.*, 1987ab, 1990; Mafu *et al.*, 1991). Several studies have shown reduced bacterial adhesion to correlate with reduced substratum hydrophobicity (Fletcher & Loeb, 1976, 1979; Pringle & Fletcher,

1983; Bidle, Wickman & Fletcher, 1993). Fletcher & Loeb (1976, 1979) reported that a marine pseudomonad exhibited maximal attachment to hydrophobic surfaces and lower attachment to hydrophilic surfaces. Pringle & Fletcher (1983), using a range of freshwater bacteria, did not reveal any generic pattern of attachment to test surfaces, although the majority of isolates showed a preferential attachment to hydrophobic surfaces. Increased adhesion to hydrophobic surfaces does not occur for all microorganisms, for example, Busscher *et al.* (1990) found optimal adhesion of *Streptococcus sanguis* to surfaces of reduced hydrophobicity whilst Denyer *et al.* (1993) found optimal adhesion of *Staphylococcus epidermidis* to polystyrene surfaces occurred over a specific hydrophobicity range.

Mafu *et al.* (1991) reported that *L. monocytogenes* Scott A showed greater attachment to hydrophobic surfaces (polypropylene and rubber) than to hydrophilic surfaces (glass and stainless steel) by using contact angle measurement. Similarly, Al-Makhalafi, McGuire & Daeschel (1994) showed that adhesion of *L. monocytogenes* to hydrophobic silica surfaces was higher than to hydrophilic silica surfaces. Results in this study have also shown a strong positive correlation between increasing substratum hydrophobicity (measured using advancing water contact angles) and adhesion of *L. monocytogenes* to model surfaces (Section 3.4.3.ii.c). Untreated glass coverslip surfaces were relatively hydrophilic, however, an increased proportion of hydrophilic to hydrophobic groups on glass surfaces could be achieved by flame oxidation of the surface which consequently resulted in lower adhesion levels. Similarly chloroform washing of glass increased the proportion of hydrophobic to hydrophilic groups; this correlated with an increase in the adhesion level obtained. The increases in adhesion levels to glass surfaces following the above treatments was not due to surface roughness since roughness measurements of Rz, Rpm and Ra on the three surface types were not significantly different.

Adhesion of *L. monocytogenes* to hydrophobic dental wax was reduced when dimethyl sulfoxide (DMSO) was added to the cell suspending medium (Section 3.6.9). Absolom *et al.* (1983) also showed that adhesion of *L. monocytogenes* to hydrophobic surfaces was decreased with the addition of various levels of DMSO to Hank's balanced

salt solution. Absolom *et al.* (1983) showed that adhesion of *L. monocytogenes* to hydrophobic surfaces was higher than to hydrophilic surfaces when the liquid surface tension was high (low DMSO concentration), however, adhesion to hydrophilic surfaces was higher than to hydrophobic surfaces when the liquid surface tension was low (i.e high DMSO concentrations). Although adhesion to hydrophilic surfaces did increase slightly at high DMSO concentrations, the higher adhesion to hydrophilic than hydrophobic surfaces under these conditions was principally the result of a dramatic decrease in adhesion levels to the hydrophobic surface. This is in general agreement with the predictions of the Wetting Theory which predicts adhesion will occur if it results in a decreased total free energy of the system. In essence the theory predicts that adhesion is more extensive to hydrophilic substrata (i.e. high surface tension τ_{SV}) than to hydrophobic substrata (low τ_{SV}), when the surface tension of the bacteria τ_{BV} is larger (i.e. hydrophilic cells) than that of the suspending medium (high DMSO concentration decreases liquid surface tensions τ_{LV}). When the surface tension of the suspending liquid is larger than that of the bacteria the adhesion to hydrophobic surfaces is more extensive than to hydrophilic surfaces (Absolom *et al.*, 1983). Therefore, by accounting for all components of the system, different degrees of bacterial attachment are observed for different relationships between τ_{SV}, τ_{LV} and τ_{BV} (Fletcher & Pringle, 1985). Consequently, although DMSO was shown to reduce adhesion levels on dental wax surfaces, its effect on adhesion to hydrophilic surfaces may be quite the opposite. It is not expected, however, that adhesion levels to hydrophilic surfaces will reach levels as high as those observed on hydrophobic surfaces in this study.

The fact that DMSO affected the adhesion of *L. monocytogenes* to dental wax does, however, confirm that hydrophobic interactions are of prime importance in its adhesion to surfaces. The above concept may also explain the observation that *L. monocytogenes* adhesion to dental wax was reduced when the cells were suspended in solutions containing biological surfactants (Section 3.6.10.i). Eight biological surfactants were shown to reduce adhesion levels to dental wax by at least 75% at 100 ppm concentrations and two, namely, Brij 56 and CHAPS reduced adhesion levels by this at least 75% at 50 ppm concentrations. Future research should examine whether these

surfactants decrease or actually increase the adhesion of *L. monocytogenes* to hydrophilic surfaces as this has important industrial implications particularly when hydrophilic surfaces such as stainless steel are cleaned regularly with surfactants.

4.3.3.ii Substratum roughness and topography.

Results presented in this study showed good correlation between adhesion levels to model surfaces and the surface roughness measurement Ra. Surface roughness may affect adhesion in two ways. Firstly, a rough surface may offer a larger surface area for attachment than a smooth surface and consequently offer more adhesion sites. Secondly, a rough surface may offer greater protection from washing shear forces than a smooth surface. Distinguishing between these two effects on adhesion is, however, often extremely difficult as experiments which measure adhesion levels to surfaces also incorporate a washing procedure to remove the weakly adhered cells.

Several researchers have shown increased adhesion levels to intra-oral surfaces following surface roughening. Swartz & Philips (1957) demonstrated that when teeth were suspended in bacterial cultures, a 10 fold increase in adherent viable bacteria was observed after surface roughening. Moreover, *S. mutans* was found to adhere more frequently to rough cements than to filling materials that were highly polished (Einwag *et al.*, 1990). Furthermore, when the adhesion of *S. sanguis* to composite materials of comparable roughness was examined, only negligible differences were registered. These studies indicate a positive correlation between surface roughness and initial bacterial adhesion.

Numerous other studies have examined the effect of surface roughness on adhesion and stagnation for oral bacteria to intra-oral surfaces (reviewed by Quirynen & Bollen, 1995). SEM studies have revealed that initial colonization of enamel surfaces starts from surface irregularities such as cracks, grooves or abrasion defects. This has been explained by considering that the bacteria in these sites are sheltered from the constant shear forces produced in the mouth through processes of chewing, swallowing etc. Thus, at these sites the change from reversible to irreversible attachment can be established more easily and thus more frequently. With time, plaque areas develop at

the irregularities which alternate with less extensive colonized surrounding areas (Nyvad & Fejerskov, 1987). Similar observations have been recorded for the colonization of the fitting surface of acrylic dentures (Morris *et al.*, 1987).

Taylor & Holah (1996) noted that the size and type of surface irregularities is important in determining the effect on cleanability, with pores, jagged edges and crevices retaining bacteria after cleaning, whilst channels with smooth edges result in bacteria being flushed out by the cleaning fluid. Wirtanen *et al.* (1995) found that surface roughness was the most important factor in cleaning biofilms from stainless steel surfaces.

Surface roughness may encompass both large scale topographical and microscopic surface irregularities. Chloroform-washed glass and Spurr resin surfaces were found to have similar surface topography and hydrophobicity. Because of the similarity in surface topography these surfaces should provide similar protection to bacteria from shear forces of washing (i.e. low protection). However, Spurr resin surfaces were rougher at the microscopic level (determined from Ra measurement and SEM analysis) than glass. Therefore, the higher adhesion to Spurr resin than to chloroform washed glass was probably the result of the increased surface area for attachment on Spurr resin surface and not due to the increased protection from shear forces.

Dental wax surfaces showed an undulating topography with surface depressions measuring approximately 19 μm (determined from Rz measurements) but were also rough or pitted when observed using SEM analysis. Thus, compared to a surface such as glass which has a flat topography and is not pitted, dental wax may increase the potential for *L. monocytogenes* to adhere through its increased surface area and increased protection from washing procedures. Interestingly, it was shown that adhesion to dental wax always occurred in the pocket and valley areas of the surface and not on the raised peak areas even though the microscopic roughness of both of these sites was the same. This suggests that surface topography is more important than microscopic surface roughness in the adhesion of *L. monocytogenes* to this surface.

The extent to which the depressions on dental wax surfaces became filled with

adhering bacteria increased when the ionic concentration of the medium was increased. This may be explained by considering the strength with which the bacteria bind to the surface in different electrolyte environments. At low electrolyte concentrations, the strength with which the bacteria adhere will be weak because the bacteria can not approach the surface close enough to form an irreversible bond. Thus, adhering bacteria will remain on the surface only where protection from shear forces is very high (i.e. in pockets). When electrolyte concentrations are high, then large numbers of cells may form strong irreversible bonds with the surface and consequently these bacteria may remain adhered to the surface in high shear environments (i.e. higher on the peak to valley transect). This concept may also be used to explain the localisation of *L. monocytogenes* to cell margins of lettuce leaf surfaces. i.e. bacteria in the margins are more protected from shear forces than adhering cells which are located on the raised bulk surface of the leaf cells.

The high levels of adhesion to cabbage-wax coated surfaces (CBW) could not be correlated with the presence of topographical surface irregularities as the surface was comparable in this respect to glass and Spurr resin surfaces. In addition, the high adhesion levels to CBW surfaces could not be explained by microscopic surface irregularities, as the surface had a lower Ra value than Spurr resin surfaces, but a higher adhesion level. This implies that adhesion to CBW surfaces was not primarily governed by surface roughness but was affected strongly by other surface properties, for example, surface hydrophobicity or charge.

4.4 Cooperative mechanisms of adhesion.

In addition to bacterial cell/substratum physicochemical interactions, adhesion kinetics may also be influenced by mutual interactions between adhering bacteria and bacteria in suspension. For example, lateral attraction (positive cooperativity) or repulsion (negative cooperativity) may exist between adhering cells (Sjollema *et al.*, 1990). Lateral attraction is influenced by non-uniform distribution whereas in the case of lateral repulsion the spatial distribution is more uniform with relatively large inter-bacterial distances (Sjollema *et al.*, 1990). A Scatchard plot with a continuously

changing negative slope suggests either that the sites are all different (or heterogenous) or that the filling of one site reduces the probability that other sites will be filled. This is one definition of negative cooperativity. A Scatchard plot with a positive slope suggests positive cooperativity, where the filling of one site makes the filling of other sites more energetically favourable. Some Scatchard plots may have multiple positive and negative slopes, suggesting alternative cooperative effects.

Positive cooperativity is a mechanism which microorganisms can utilize to colonize a surface (van der Mei *et al.*, 1993). Doyle, Nesbitt & Taylor (1982), in considering the mechanism of adhesion of *Streptococcus sanguis* to saliva-coated hydroxyapatite, described positive cooperativity as the capacity of initial bound cells to change the available substratum surface in their immediate surroundings, thereby creating new receptor sites for other cells. Since the introduction of the concept of positive cooperativity, cooperative phenomena have been described or deduced for a number of bacteria including *Actinomyces viscosus* to saliva-coated hydroxyapatite, *Chlamidia trachomatis* to HeLa cells, *Escherichia coli* to BHK tissue culture cells, *Haemophilus parainfluenza* to saliva-coated hydroxyapatite and *Pseudomonas fluorescens* to glass (Reviewed by Doyle, 1991). Van der Mei *et al.* (1993) suggested that on inert substrata, positive cooperativity may result if bound cells excrete biopolymers which adsorb to the substratum forming a conditioning layer, thereby making the micro-environment of the cell more favourable for subsequent adhesion. Alternatively, suspended cells may be attracted more towards an adhering cell than to other suspended cells which may also be seen as positive cooperativity.

In this study positive cooperativity was demonstrated for *L. monocytogenes* adhesion to both lettuce leaf surfaces and dental wax surfaces (Sections 3.2.3 and 3.6.2) at low adhesion levels, this was followed in both case by a transition into negative cooperativity at intermediate adhering cell densities. For dental wax surfaces, the transition from positive to negative cooperativity occurred at a binding concentration of between approximately 4.0×10^6 and 1.0×10^7 for cells suspended in three ionic solutions (Section 3.6.2). This range corresponds to the adhesion levels at which microcolony formation in valley and pocket regions on the surface was observed using

SEM analysis (Section 3.5.4, Plate 3.20 a-g). Adhesion experiments over a 48 h incubation period (Section 3.5.1) showed that the first (or pioneering) bacterial cells to become irreversibly adhered, did so in the deepest valley and pocket areas of the surface (micrographs not shown) and that subsequent adhesion occurred in the immediate vicinity of the pioneering cells. It appears likely, therefore, that the adhesion of the pioneering cells to the surface aided the adhesion of subsequent cells through positive cooperative mechanisms. Consequently, as more cells adhered around the immediate vicinity of the pioneering cell, micro-colonies were formed and valley areas of the surface became filled. Negative cooperativity occurs on dental wax surfaces because as the adhesion sites lower on the valley to peak transect are filled, subsequent adhering bacteria are forced to adhere to less favourable sites on the substratum. Thus, the filling of sites low on the peak to valley transect may interfere with the filling of additional sites due to the physical heterogenicity of the surface. Negative cooperativity will occur at lower adhesion levels for cells suspended in low ionic solutions than in high ionic solutions. This is because increasing electrolyte concentration will allow the bacteria to form stronger irreversible bonds with the surface and, therefore, adhere to sites higher on the substratum. Effectively, increasing the ionic strength of the medium in which the cells are suspended indirectly increase the number of binding sites available for bacterial adhesion.

The above concept may also be applied to explain the localisation of *L. monocytogenes* in the cell margins of lettuce leaf tissue. Positive cooperativity was demonstrated for lettuce tissue at low adhesion levels, possibly indicating that the filling of initial adhesion of bacteria to leaf cell margins made adhesion of subsequent bacteria to these areas more energetically favourable. Positive cooperativity was followed by negative cooperativity possibly as the adhesion sites in the cell margins became filled. Advancing water contact angle measurements showed that a relatively low number of hydrophobic groups were present on lettuce leaf tissue as compared to cabbage leaf tissue (advancing water contact angles were higher for cabbage than lettuce). The higher substratum hydrophobicity of cabbage compared to lettuce may explain the differences in the distribution of adhering cells between the two tissue types and the increased

adhesion levels observed on cabbage leaf surfaces. The relatively high hydrophobicity of cabbage probably originated from the large number of natural wax deposits which were present on the substratum. Thus, in addition to the leaf cell margins of cabbage tissue, additional adhesion sites i.e. the wax deposits, were available for the bacteria to form strong irreversible bonds to. Consequently, the distribution of adhering cells on cabbage tissue may be quite different to the distribution of cells on lettuce tissue because of differences in the numbers and type of adhesion sites available on these leaf surfaces.

Localisation of bacterial cells to leaf cell margins has also been shown by Wallace & Perombelon (1993) who studied adhesion of *Erwinia carotovora* to the leaflets of potato plants. They suggested that it was unlikely that this was due to insufficient washing as increasing the number of washes did not significantly decrease the numbers of bacteria recovered from the leaves. However, Haahtela *et al.* (1989) found that *Erwinia carotovera* adhering to leaf surfaces of *Nicotiana tobacum* were not localised to leaf cell margins but distributed over the entire surface of the leaf. Thus, as with *L. monocytogenes* in this study, the distribution of adherent *Erwinia carotovera* on leaf surfaces may depend on the leaf surface used. Wallace & Perombelon (1993) mentioned that this difference may have been the result of a different distribution of adhesion sites on the two tissue types.

Re-inoculating dental wax surfaces which had been previously saturated with irreversibly adhered *L. monocytogenes* cells (Section 3.6.5) did not increase adhesion levels significantly. This illustrates that maximum adhesion levels were dependent on the number of adhesion sites available and not on the number of potentially adherent cells that were present in the planktonic phase.

Cooperative adhesion mechanisms were not investigated for cabbage tissue, however, SEM analysis did indicate that adhering cells often formed micro-colonies around leaf wax deposits possibly indicating that the bacteria were adhering to these deposits through positive cooperativity. Future studies should investigate the presence or absence of cooperative mechanisms for cabbage tissue. If positive cooperativity only exists for cells adhering to wax deposits then the adhesion level at which positive cooperativity becomes negative will ultimately be dependent on the number of wax

deposits present on the surface. Jeffree *et al.*, 1975 showed that the integrity of leaf wax deposits degrades throughout the growing season. Consequently, if adhesion levels to cabbage tissue are dependent on the number of wax deposits present and the ability of the bacteria to adhere under positive mechanisms, then adhesion levels to cabbage tissue may vary considerably over the growing and storage periods. Therefore, future studies should also compare adhesion of *L. monocytogenes* to cabbage tissue at different time points in the growth and storage of this vegetable.

The mechanism of positive cooperativity for *L. monocytogenes* remains unclear. Results showed, however, that maximum adhesion of U.V. irradiated *L. monocytogenes* cells to dental wax was significantly lower than adhesion levels of viable cells (Section 3.5.3). Thus, although U.V. irradiated cells were capable of adhering to the surface, possibly simply through strong physico-chemical interactions, subsequent adhesion involving microcolony formation did not occur, possibly because the non-viable cells could not interact actively through cooperative mechanisms. The maximum adhesion level for U.V. irradiated cells was above 5.0×10^5 but below 1.0×10^6 cells cm^{-2} (Fig. 3.18). It is between these adhesion levels that micro-colony formation is initiated (see Plate 3.20 b & c). It is possible that the inability to form microcolonies was simply because initial adhering cells, which adhered to the surface through generic physicochemical bonding could not produce an extracellular polymer once adhered and could not, therefore, condition their surrounding environment sufficiently to allow subsequent bacteria to adhere.

It is clear that by considering the chemical and/or physical heterogenicity of a substratum and the presence of cooperative adhesion mechanisms, the different distribution that adhering cells exhibit on different surfaces may be explained. For lettuce tissue, dental wax surfaces and cabbage wax-coated (CBW) surfaces, adhering cells were distributed in non-uniform patterns. i.e. either localised to surface depressions such as leaf cell margins or valley areas, or forming small microcolonies. The adhesion sites on dental wax and lettuce leaf surfaces are physically heterogenous due to the topography of these surfaces. Thus, localisation of bacteria to surface depressions may have occurred because these sites provide a more favourable physical environment for

adhesion than the more exposed sites. Similarly, CBW surfaces were shown to be chemically heterogenous, consisting of high numbers of both hydrophobic and hydrophilic sites (as determined from contact angle measurements). The distribution of these different hydrophobic and hydrophilic sites on the substratum could not be determined. It is, however, possible that the distribution of adhering bacteria was directly related to the distribution of these chemical groups. For example, if the hydrophobic sites were localised to discrete areas on the substratum, then if the bacteria only adhered to these hydrophobic sites they would also be localised to certain areas on the substratum.

4.5 Conclusions

The adhesion of *L. monocytogenes* to leaf and model surfaces is strongly influenced by the electrolyte composition of the medium in which the cells are suspended. High electrolyte concentrations may increase adhesion levels by decreasing the electrostatic repulsion forces which exist between the bacterium and the substratum. This occurs as a result of an increased compression of the diffuse double layers surrounding both surfaces which effectively allows more bacteria to approach the surface to close enough separation distances to form an irreversible bond. These findings are consistent with the predictions of the DLVO theory.

The majority of *L. monocytogenes* cells in suspension expressed a net hydrophilic nature and a minority expressed a net hydrophobic nature. The proportion of hydrophobic to hydrophilic cells increased in the presence of monovalent and divalent electrolytes. It is not clear, however, whether increasing cell surface hydrophobicity increases the capacity of these bacteria to adhere to leaf and model surfaces. However, increasing substratum hydrophobicity of model surfaces correlated strongly with increasing adhesion levels indicating that the formation of hydrophobic bonds was an important factor in *L. monocytogenes* adhesion. Furthermore, adhesion to hydrophobic dental wax surfaces was reduced significantly when the surface tension of the cell suspending medium was decreased by the addition of DMSO. This confirms the importance of hydrophobic interactions in *L. monocytogenes* adhesion to this surface.

Substratum topography and microscopic roughness strongly influenced the maximum adhesion levels reached on model surfaces. Surface topographical depressions may provide protection from shear forces, and consequently increase the number of adhesion sites available to the bacterium to form strong irreversible bonds. The number of sites which are filled is dependent on the strength with which the bacteria can bind to these sites. Again high electrolyte concentrations can increase the filling of these sites by eliminating barriers to adhesion, i.e by reducing electrostatic repulsion and increasing hydrophobic bonding. Adhesion of *L. monocytogenes* to surfaces with high microscopic surface roughness (e.g. Spurr resin) was higher than to surfaces with low microscopic roughness (e.g. CW glass) of similar surface topography and hydrophobicity. Thus, microscopic surface roughness is believed to favour adhesion, possibly by offering the bacteria an increased surface area for bond formation. However, surface topography appeared to affect adhesion more strongly than microscopic roughness for surfaces such as dental wax and lettuce leaf tissue.

Adhesion of *L. monocytogenes* to leaf and model surfaces is, ultimately determined by the inter-relationship of the physico-chemical properties of the suspending medium, substratum and the cell surface. In food hygiene environments control of these parameters is limited. However, this study has shown that strategies which ensure a smooth surface topography and low substratum hydrophobicity will reduce *L. monocytogenes* adhesion. Additionally, the development of washing procedures which utilise a combination of chelating agents and surfactants may reduce adhesion through suppressing attractive physico-chemical interactions.

Chapter Five

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Appendices

Appendix A: Microbiological media.

A.1 Listeria selective agar base (Oxoid CM 865), plus Listeria selective supplement (SR140) (Oxford Formula) (LSA).

Agar Base Formula	(grams per litre)
Columbia Blood Agar Base	39.0 g
Aesculin	1.0 g
Ferric Ammonium Citrate	0.5 g
Lithium chloride	15.0 g
final pH 7.0 ± 0.2	

Selective Supplement Formula	(per vial)
Cyclohexamide	200 mg
Colistin sulphate	10.0 mg
Acriflavine	2.5 mg
Cefotetan	1.0 mg
Fosfomycin	5.0 mg

Each vial is sufficient to supplement 500 ml of medium.

Directions:
Suspend 27.75 g of Listeria selective agar base in 500 ml of distilled water. Sterilise by autoclaving at 121°C for 15 min. Cool to 50°C and aseptically add the contents of one vial of Listeria selective supplement reconstituted with 5ml ethanol/sterile distilled water (1:1). Mix well and pour into sterile petri dishes.

A.2 Tryptone soy agar (Oxoid CM131)

Formula	(per litre)
Tryptone	15.0 g
Peptone	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Final pH 7.3 ± 0.2	

Directions:
Mix 40.0 g of the solids with 1 litre of distilled water. Sterilise by autoclaving at 121°C for 15 min. Mix well and pour into sterile petri dishes.

A.3 Tryptone soy broth (Oxoid CM129B) (TSB)

Formula	(per litre)
Pancreatic digest of casein	17.0 g
Papayic digest of soybean meal	3.0 g
Sodium chloride	5.0 g
di-basicpotassium phosphate	2.5 g
Glucose	2.5 g
Final pH 7.3 $\pm$ 0.2	

Directions:

Mix 27.5 g of the solids with 1 litre of distilled water. Sterilise by autoclaving at 121°C for 15 min. Mix well and dispense aseptically as required.

A.4 Brain Heart Infusion broth (Oxoid CM225)

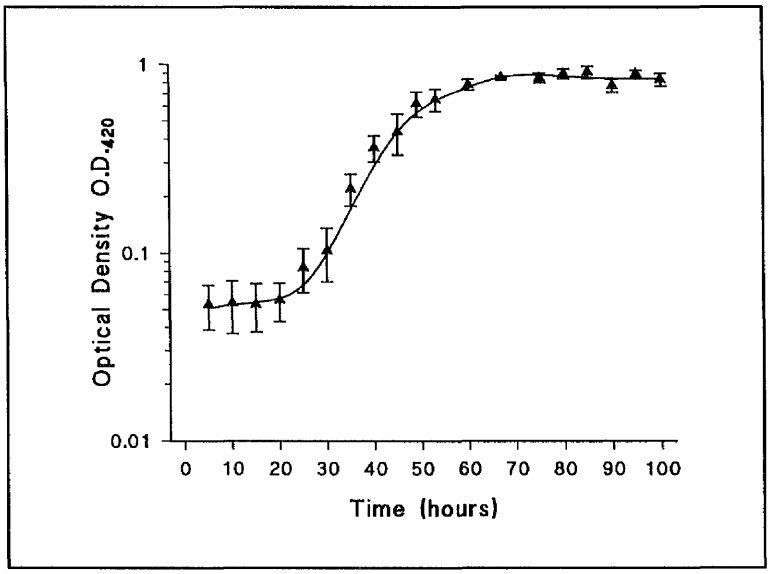
Formula	(per litre)
Calf brain infusion solids	12.5 g
Beef heart infusion solids	5.0 g
Proteose peptone	10.0 g
Glucose	2.0 g
Sodium chloride	5.0 g
di-sodium phosphate	2.5 g
Final pH 7.3 $\pm$ 0.2	

Directions:

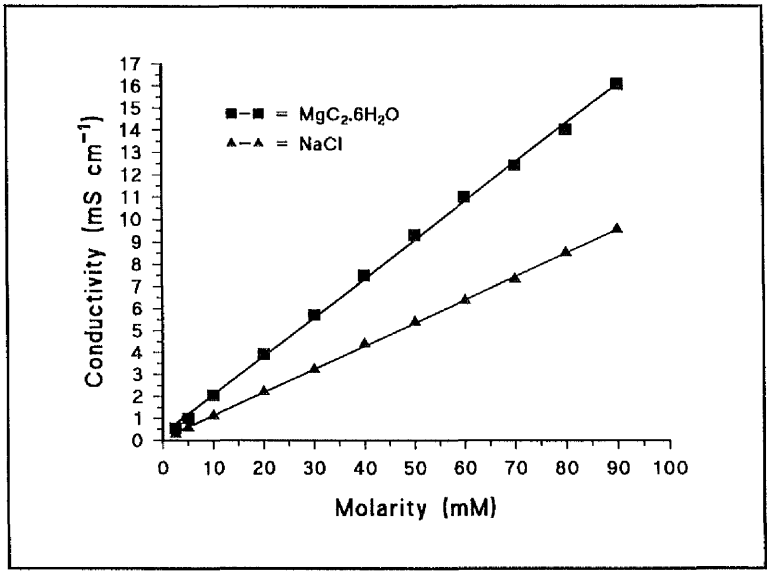
Mix 29.5 g of the solids with 1 litre of distilled water. Sterilise by autoclaving at 121°C for 15 min. Mix well and dispense aseptically as required.

Appendix B: Standard curves.

B.1 Growth curve for *L. monocytogenes* CRA 433 in Tryptone Soy broth at 10°C.



B.2 Conductivity measurement of ionic solutions.



Solutions were prepared in sterile deionized water (Milli-Q reagent grade water systems; Millipore corporation, Mass.) pH 7.1.