STUDIES ON THE RAPID DETECTION OF THE FOOD-BORNE
PATHOGEN <u>Listeria monocytogenes</u>.

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ABSTRACT

In this work a selective impedimetric assay was developed with the aim of reducing the presumptive detection time of the pathogenic foodborne microbe <u>Listeria monocytogenes</u>.

For rapid detection of the target organism, the physical assay conditions and the nutritional composition of the impedimetric medium were optimised for the metabolism of <u>L. monocytogenes</u>. Impedance, the algorithm found to be most reliable and reproducible, was used to monitor the electrical responses of the target species and impedance magnitude and detection time were chosen as the signals for detection.

Antimicrobial agents were screened for their ability to eliminate the electrical signals produced by a range of bacterial and fungal species. The combination of 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam were found to permit the sole detection within 36 h of low concentrations of <u>L. monocytogenes</u>, <u>L. innocua</u> and <u>L. welshimeri</u>. The electrical responses of these three organisms were delayed minimally by the selective agents at concentrations used. Temperature stressed cells of <u>L. monocytogenes</u> could be detected without a non selective enrichment stage.

Two impedimetric procedures were developed for cheese suspensions. A direct assay, capable of enumerating the target species at concentrations greater than 10^2 CFU g^{-1}, and an assay for detecting very low levels of <u>L. monocytogenes</u> (down to 10 CFU per 25 g) after 24 h of selective enrichment. These assays are economical in materials and are low in labour intensity. As traditional selective medium constituents are used, the safety of the assays is at least comparable with current techniques.

No false negative results were obtained in this work. However, a number of types of artefactual curves were recognised. The quality of electrical signals produced by <u>L. monocytogenes</u> was found to be influenced by selective agents and by the chemical components and normal microflora of foodstuffs. For example, lithium chloride increased curve magnitude, while potassium tellurite decreased this parameter. Detection time was found to be much less affected by external factors and was therefore used as the major criterion for detection.

The biochemical basis of the impedimetric detection of <u>L. monocytogenes</u> was investigated. While some success was obtained with detecting lactic acid and ammonia using commercially available test kits, problems were encountered with the quantification of low levels of organic acids by gas chromatography. However, it was argued that the primary metabolites likely to contribute to the impedimetric detection curve of <u>L. monocytogenes</u> are L-lactic acid and ammonium ions.

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ABBREVIATIONS

ANB   aesculin nutrient broth
AOAC   association of official analytical chemists
Apr   April
ATCC   American Type Culture Collection
ATP   adenosine triphosphate
Aug   August
BS   British standard
BUCO   arbitrary units of conductance change
BUCA   arbitrary units of capacitance change
BUIC   arbitrary units of impedance change
C   capacitance
CFU   colony forming unit
COSSH   control of substance hazardous to health
Cpol   polarisaion capacitance
Dec   December
DIFT   direct immunofluorescent test
DNA   deoxyribonucleic acid
DT   detection time
DTH   delayed type hypersensitivity
E   voltage
EIA   enzyme immunoassay
ELISA   enzyme linked immunosorbent assay
EMP   Embden Meyerhof Parnas
Fig.   figure
GNB   glucose enriched nutrient broth
G   conductance
h   hour
HACCP   hazard analysis / critical control point
I   current
ISA   immunosuppressive activity
Jan   January
Jul   July
Jun   June
LPM   lithium chloride, phenylethanol, moxalactam
Ltd   limited
Mar   March
MPA   monocytosis producing agent
mol. wt.   molecular weight
NAD   nicotinamide adenine dinucleotide
NADase   nicotinamide adenine dinucleotidease
NADH   reduced nicotinamide adenine dinucleotide
NCIB   National Collection of Industrial Bacteria
NCTC   National Collection of Type Cultures
NH_4^+ ammonium ion
NK   not known
No.   number
Nov   November
NS   not stated
NT   not tested
Oct   October
PCR   polymerase chain reaction
PRR   peak response rate
PRT   peak response time
r   correlation coefficient
R   electrical resistance

RABIT	rapid automated bacterial impedance technique
RIA	radioimmunoassay
RNA	ribonucleic acid
RNB	L-rhamnose nutrient broth
Rpol	polarisation resistance
Rsol	bulk fluid resistance
SD	standard deviation
Sep	September
subsp	subspecies
t	t-Test Value
TVC	total viable count
UHT	ultra heat treatment
UK	United Kingdom
US	United States of America
USDA	United States Department of Agriculture
WHO	World Health Organisation
z	impedance

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Persius, 34-62 AD:

Venienti occurrite morbo

CHAPTER 1

INTRODUCTION

1.1 Review of microbial food poisoning

Microbial food poisoning is responsible for major economic losses; in the United States (US) it is second only to the common cold in causing absence from work (Madden et al., 1986). Food forms the bulk of foreign material encountered by people every day. In many situations it probably has a passive role in transmission of pathogens, the microorganisms not growing in the food, but only being transmitted by it (Archer & Young, 1988). Traditional symptoms of food poisoning include diarrhoea and vomiting, but other symptoms can occur and in severe cases death can result (Kvenberg, 1988). In England and Wales the incidence of microbial food poisoning has increased 300 % between 1981 and 1990 (Adams & Roper, 1990). Both bacteria and viruses have been implicated in its causation.

1.1.1 Bacterial food poisoning

Foodborne bacterial illnesses can be subdivided into two types, infections and intoxications. The first type of illness is caused by ingestion of the causative microbe which then either invades the intestinal epithelia or produces toxins. Examples include food poisoning caused by Listeria monocytogenes, Salmonella typhimurium, Yersinia enterolitica, Campylobacter jejuni,

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Vibrio parahaemolyticus and Clostridium perfringens (Archer & Young, 1988; Dirksen & Flagg, 1988, Madden et al., 1986). In the second type the presence of viable microorganisms in the food is not necessary as the illness is caused by the ingestion of a preformed toxin. Examples include food poisoning caused by Cl. botulinum, Bacillus cereus and Staphylococcus aureus.

Archer & Young (1988) reported that while Salmonella spp. produced the largest number of cases of food poisoning in the US and other industrialised nations during the last decade, L. monocytogenes caused the outbreak with the highest mortality. Listeria, responsible for 291 cases of listeriosis and 63 deaths in England and Wales in 1988 (Adams & Roper, 1990), will be discussed in detail in Section 1.2.

The salmonellae are small, Gram negative, nonsporing rods. They are widely distributed in nature, with the intestinal tract of animals and humans being their primary reservoirs. S. typhimurium and S. enteritidis are the most commonly found foodborne serovars. The virulence of salmonellae possibly involves two toxins, an enterotoxin and a cytotoxin (Jay, 1992). In 1989 25,000 cases of salmonellosis occurred in England and Wales (Adams & Roper, 1990).

Yersinia spp. are found in the intestinal tract and faeces of animals and in raw and pasteurised milk. While Y. pestis was the cause of the plague, the species of interest in foods is Y. enterocolitica. Ingestion of this organism can cause the typical symptoms of

gastroenteritis (Dirksen & Flagg, 1988).

Y. enterocolitica produces a heat stable enterotoxin that survives at 100 °C for 20 minutes, although virulence appears to be a result of tissue invasiveness (Jay, 1992). There were 580 cases of Y. enterolitica infections in England and Wales in 1989 (Waites & Arbuthnott, 1991). Yersinia is an emerging pathogen, its true importance has only begun to be realised (Archer & Young, 1988).

Campylobacter spp. can be found in raw milk, unchlorinated drinking water, undercooked chicken, raw shellfish and mushrooms (Dirksen & Flagg, 1988). The species of primary importance in foods is C. jejuni. It is a spirally curved, microaerophilic Gram negative rod. C. jejuni produces a heat labile enterotoxin that is similar in some properties to the enterotoxins of the water borne pathogens V. cholerae and E. coli, the causal agents of cholera and tourists diarrhoea respectively (Jay, 1992). Campylobacter food poisoning is now the commonest form of infective diarrhoea in most developed countries with incidence exceeding that of salmonellosis (Skirrow, 1991; Baggerman & Koster, 1992). In 1989 approximately 32,000 cases occurred in England and Wales (Adams & Roper, 1990; Baggerman & Koster, 1992).

Vibriosis, a gastroenteric syndrome caused by Gram negative V. parahaemolyticus, is contracted almost solely from seafood. V. parahaemolyticus produces a heat stable haemolysin which correlates to pathogenicity. Infection by this organism results in mild symptoms, hospitalisations being rare. Over the last 20 years

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environmental strains of _Vibrio_ spp. have become established as pathogens of man. In the United Kingdom (UK) vibriosis is relatively infrequent compared to other developed countries such as the US, infections mainly being carried into the country from abroad (West, 1989).

Three Gram positive spore-forming rods are known to cause bacterial food poisoning: _Cl. perfringens_, _Cl. botulinum_ and _B. cereus_ (Jay, 1992). Large numbers of viable _Cl. perfringens_ cells must be ingested to cause symptoms (Jay, 1992). There are two different types of food poisoning associated with this organism: type A is a mild form of food poisoning with classic symptoms, whilst type C is more severe and is known as necrotic enteritis (Granum, 1990). Due to the mildness of type A poisoning the true incidence of _Cl. perfringens_ enteritis has been said to be unknown (Granum, 1990), however Waites & Arbuthnott (1991) stated that the number of reported cases in the UK increased by 46% between 1986 and 1988 and in the latter year 1312 cases were recorded (Waites & Arbuthnott, 1991).

Botulism is caused by the anaerobic sporeformer, _Cl. botulinum_. Some of the symptoms, which develop between 12 and 72 h after ingestion of the toxin-containing food, include double vision, vomiting, fatigue, dizziness, headache, paralysis of muscles, and finally respiratory failure and death. The mortality rate is variable between 30 and 65%, depending on host susceptibility (Jay, 1992). Although _Cl. botulinum_ is rare in the UK, an outbreak, associated with the

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consumption of hazelnut yogurt, occurred in 1989 consisting of 27 cases with one fatality. In the previous 10 years only one case had been reported (Waites & Arbuthnott, 1991).

B. cereus, an aerobe normally present in soil, dust and water, has been known as a food poisoning organism in Europe since at least 1906. Symptoms are caused by two enterotoxins, a diarrhoeagenic toxin and an emetic toxin (Shinagawa, 1990; Jay, 1992). There were 110 reported outbreaks of B. cereus food poisoning in the UK between 1971 and 1978 (Shinagawa, 1990). More recently incidence of Bacillus spp. infection in the UK has been reported to be low. In 1986 there were 65 cases and in 1987 137 cases. However in 1988 an increase to 418 cases was reported. Most of these incidents of food poisoning were caused by B. cereus (Waites & Arbuthnott, 1991).

Staphylococci can be expected to occur in all food products of animal origin or foods handled directly by humans. A high percentage of people are carriers of Staph. aureus, with approximately 50% of adults harbouring the organism in the nasal cavity (Jay, 1992). The five proteinaceous toxins of Staph. aureus are to some degree heat resistant and therefore need thorough heat processing for degradation. In 1989 there were 100 reported cases of Staph. aureus intoxication in the UK (Waites & Arbuthnott, 1991).

1.1.2 Viral food poisoning
Foodborne viral gastroenteritis was first recognised in

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1968 in Norwalk, US (Halligan, 1992). The most common sources of infection are shellfish, due to these animals concentrating particles during filter feeding (Jay, 1992), and foods that have been contaminated by food handlers (Halligan, 1992).

There are two main types of foodborne virus, the Norwalk type virus and hepatitis A (Halligan, 1992). In the UK approximately 90 % of reported outbreaks of viral gastroenteritis are instigated by the former virus (Halligan, 1992) which is endemic here (Waites & Arbuthnott, 1991). The main characteristic of infection by the Norwalk agent is uncontrollable vomiting within 36 hours (h) of contact with the contaminated foodstuff (Halligan, 1992). The first confirmed outbreak of foodborne Norwalk gastroenteritis in the UK occurred in the South of England in 1976 and 1977 and was associated with contaminated cockles. In 1989 only 379 cases of this and similar types of viral infection were reported, possibly due to the difficulty of virological investigation (Waites & Arbuthnott, 1991).

The main symptom of hepatitis A infection is jaundice which takes 4 to 6 weeks to develop. The virus is therefore very difficult to control amongst food handlers (Halligan, 1992). An outbreak of hepatitis A occurred between 1989 and 1991 in Gloucester where 162 cases were identified (Majeed et al., 1992). A large number of subclinical infections must also occur, however, as there is serological evidence of infection by hepatitis A in 20 to 30 % of young adults (Waites &

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Arbuthnott, 1991).

1.2 Review of Listeria and listeriosis

1.2.1 Characteristics of L. monocytogenes

L. monocytogenes was first described in 1926 by Murray et
al. who reported an epidemic in laboratory rabbits and
guinea pigs. Infection was caused by a microbe they
called Bacterium monocytogenes due to its production of
mononuclear leukocytes in the laboratory animals. In 1927
Pirie isolated the same bacterium from infected gerbils
and named the genus to which it belonged Listerella in
honour of the surgeon Lord Lister. For taxonomic reasons
this name was later changed to Listeria (Pirie, 1940).
Listeria monocytogenes was soon proven to be a pathogen
of man. In 1942 Cotoni and Dumont identified an organism
that they had previously isolated from the cerebrospinal
fluid of a soldier in France in 1919 as L. monocytogenes
(Cotoni, 1942).

The genus Listeria was originally monotypic and
contained only L. monocytogenes (McLauchlin, 1987; Jones,
1989). By 1987, however, it had been expanded to include
8 species: L. monocytogenes, L. ivanovii, L. innocua,
L. welshimeri, L. seeligeri, L. murrayi, L. grayi and
L. denitrificans (McLauchlin, 1987). Since this time
L. denitrificans which had little to justify its
inclusion in this genus (Lovett, 1990), has been
reclassified as Jonesia denitrificans on the basis of
numerical taxonomic, biochemical, serological,
morphological and nucleic acid studies. Nucleic acid
hybridisation studies have also indicated that the genus

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Listeria has two distinct lines of descent: i)
L. monocytogenes, L. ivanovii, L. innocua, L. seeligeri
and L. welshimeri and ii) L. grayi and L. murrayi
(Rocourt, 1989).

L. monocytogenes, the main organism of study in this
research, is a Gram positive bacillus, at times appearing
as a diptheroid like rod. It is a relatively small
measuring 1.0 μm to 4.0 μm in length and 0.5 μm in width.
It has a single polar flagellum and is actively motile
between 18 °C and 22 °C with characteristic tumbling and
rotating motions. L. monocytogenes is a facultative
organism able to grow on a wide variety of substrates.
The growth of the organism is enhanced by glutamine,
glucose and ferric chloride (Kramer & Jones, 1969), serum
and blood (Leighton, 1985).

On the basis of the antigenic structure of the outer
layers of the cell L. monocytogenes has been divided into
a number of serological types (serotypes): 1/2a, 1/2b,
1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7
(Seeliger, 1984; Lovett, 1990). Serotypes 1/2, 3 and 4
have all been identified from cases of human listeriosis
in the UK between 1967 and 1984 (McLauchlin et al.,
1986). A second typing system was developed by Audurier
et al. (1979) based on the lytic properties of a set of
bacteriophages. This can be used to lysotype 57.7 % of
serotype 1/2 strains, 88 % of serotype 4 and less than
half of the other serotypes. However, due to inherent
problems in reproducibility, work is in progress to
develop other typing systems (McLauchlin, 1987).

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L. monocytogenes is capable of growth over a wide
temperature range. It is usually classified as being
psychrotrophic (Junttila et al., 1988), with optimum
growth occurring at 30 °C (Kramer & Jones, 1969;
Leighton, 1985). It can also survive heating at 50 °C for
75 minutes (Golden et al., 1988b) and Lemaire et al.
(1989) have reported that serotype 1 is significantly
more heat resistant than serotype 4. In addition
L. monocytogenes can withstand extremes of pH, growing
within the range of 5.5 to 9.5 (Wehr, 1987; George et
al., 1988).

L. monocytogenes is widespread in nature (Junttila
et al., 1988). It has been found in a variety of
environments including soil, vegetation and water from
both farms and non agricultural lands (Heisick et al.,
1989a; Skovgaard, 1989; Al-Ghazali & Al-Azawi, 1990). It
has also been cultured from healthy and diseased humans
and a variety of animals, birds, fish and crustacea
(World Health Organisation (WHO) Working Group, 1988;
Schönberg, 1989). Animals and humans can also be healthy
enteric carriers (WHO Working Group, 1988).

Ralovich (1989) investigated the virulence of a
number of strains of Listeria spp. The results of the
survey, summarised in Table 1.1, indicate that while both
virulent and avirulent strains are able to adhere and
penetrate, only the former can multiply within eukaryotic
cells. This study utilised procedures described by

Assay	Virulent Strains			Avirulent Strains		
Beta haemolysis	+	+	+	±	-	-
Antons test	+	+	+	-	-	-
Mouse test	+	+	-	-	-	-
Chick embryo test	+	+	NT	NT	-	-
Adhesion	+	+	+	NT	+	+
Penetration	+	+	+	NT	+	+
Multiplication in cells	+	+	+	NT	-	-

Key
NT = not tested

Table 1.1 Properties of Listeria strains. Each of the six columns represents the response of one or a group of strains.

Schönberg (1989). To demonstrate ß haemolysis either a simple blood agar plate is streaked or a more complicated CAMP test was carried out. The CAMP test involved streaking a culture of Staph. aureus, Rhodococcus equi or Micrococcus lylae across the centre of a blood agar plate. The Listeria strain was then streaked perpendicular to and within 3 mm of the line of the first inoculum. After overnight incubation at 37°C the plate was examined. A positive reaction was an arrowhead shaped zone of haemolysis around the test streak. Several in-vivo tests involved injecting mice and chick embryos. Only pathogenic strains of L. monocytogenes killed the test animal within 48 h. In another in-vivo test, the Antons eye test, one drop of L. monocytogenes containing

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10^6 cells was dropped into the eyes of a rabbit or guinea pig. A positive response here was the development of conjunctivitis. To test for adhesion, penetration and multiplication Ralovich et al. (1986) used light and electron microscopic studies for visual examination of the invasion of keratoconjunctival epithelial membranes by the Listeria strains. Gaillard et al. (1987a) using different techniques came to the same general conclusions as Ralovich (1989).

The pathogenic process or infection is a result of the virulence factors of a pathogen allowing it to grow at a faster rate than the death rate caused by a hosts defence mechanisms (Cowart, 1987). L. monocytogenes has many such virulence factors. The major virulence factor is a cytotoxic haemolysin, called listeriolysin O or alpha listeriolysin (Junttila et al., 1988; Schönberg, 1989; Ralovich, 1989). The existence of this extracellular haemolysin was demonstrated as early as 1941 (Harvey & Farber, 1941). Most researchers are certain there is a relationship between haemolysis and pathogenicity in the genus Listeria. All Listeria strains from patients or people that have died from listeriosis, cause ß type haemolysis on blood agar (Ralovich, 1989). Both L. monocytogenes and L. ivanovii can be haemolytic and pathogenic. Other species in the genus, L. innocua, L. welshimeri, L. grayi and L. murrayi cannot produce haemolysis and are considered to be non pathogenic (Berche et al., 1987; Cluff & Ziegler, 1987; Schönberg,

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1989). There is one exception, however, haemolytic but
avirulent L. seeligeri (Smola, 1989).

Listeriolysin O, which is very similar to
streptolysin O and pneumolysin, has a molecular weight of
58 kilo Daltans (kDa) (Flamm et al., 1989; Kathariou &
Pine, 1990), and is made up of 504 amino acids.
L. ivanovii and L. seeligeri produce thiol dependent
exotoxins that are similar but not identical to
listeriolysin O. Listeriolysin O is mainly produced in
the exponential growth phase and is active at pH 5.5 but
not at pH 7.0, suggesting a role in macrophage phagosomes
(phagocytic vacuoles) (Jay, 1992).

Gaillard et al. (1987b) determined the main function
of listeriolysin O was the disruption of membranes to
allow the bacteria to escape from the phagocytic vacuoles
of host cells to replicate in and utilise components of
the cytoplasm. Cowart (1987) suggested a second role for
listeriolysin O was in the acquisition of iron, a
limiting factor for growth of pathogens during infection.
Listeriolysin O causes the disruption of red blood cells
thereby permitting the mobilisation of iron from
haemoglobin. Cowart (1987) found that haemolysin
production in L. monocytogenes is precisely regulated by
the external iron concentration and that the organism
produces another siderophore, a soluble reductant, that
mobilises iron from transferrin in serum.

In addition to listeriolysin O and the soluble
reductant L. monocytogenes produces many more substances
which enables it to resist host defences and therefore

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could be considered to be virulence factors. Flamm et al. (1989) investigated a major secreted polypeptide that was capable of lysing erythrocytes. They found it to have a molecular weight of 60 kDa and suggested it could be ß listeriolysin, a lipase or a protease. Kuhn and Goebel (1989) also reported a major secreted polypeptide of 60 kDa, made up of 484 amino acids, which they designated P60. This factor could be involved in uptake of L. monocytogenes by non professional phagocytic cells. Notermans et al. (1989a) described a delayed type hypersensitivity factor (DTH) that significantly correlates with pathogenicity. While Hof and Chatzipanagiotou (1987) assigned this activity to a cell wall protein, Galsworthy (1987) suggested a peptidoglycan was responsible.

In the 1940's it was shown that phenol water extracts of L. monocytogenes could induce the production of monocytes (Stanley, 1949). The monocytosis producing agent (MPA) was analysed by Galsworthy (1987) and found to be an anionic material associated with the plasma membrane. It has a molecular weight of approximately 1000 and contains no amino acids or carbohydrates. The ability of a pathogen to promote the production of cells destined to destroy it appears paradoxical and the precise role of the MPA in the overall pathogenicity of L. monocytogenes remains unclear. Galsworthy (1987) also reported a immunosuppressive activity (ISA) which suppresses the antibody producing ability of spleen cells from Listeria infected animals. ISA, which is present in aqueous

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extracts of heat killed cells, has a molecular weight of
150 kDa and contains amino acids, carbohydrates,
phosphorous and glycerol. Galsworthy (1987) has suggested
that both ISA and MPA may act by confusing the production
and function of macrophages.

L. monocytogenes does not possess a capsule,
important for virulence in other bacterial species (Hof &
Chatzipanagiotou, 1987). It does, however, have a lipid
component that shares a number of similarities with the
lipopolysaccharide typical of the outer membranes of Gram
negative bacteria including pyrogenicity and lethality to
rabbits. In addition both contain acylated hydroxy fatty
acids, 2-keto-3-deoxyoctonic acid and heptose (Wexler &
Oppenheim, 1979). The lipopolysaccaride-like fraction of
L. monocytogenes is approximately 6% of the dry weight of
the cell and is associated with the plasma membrane
(Wexler & Oppenheim, 1979). It possesses low tissue
toxicity and is serologically inactive (Stanley, 1949),
but kills macrophages in vitro (Galsworthy & Fewster,
1988).

Barclay et al. (1989a; 1989b) investigated a number
of extracellular products that may also be associated
with the virulence of L. monocytogenes. They detected
extracellular lipolytic activity as well as a number of
hydrolytic activities such as phospholipase C, acid
phosphatase, neutral phosphatase, nicotinamide adenine
dinucleotidease (NADase), lipase and esterase. Although
the action of many of these substances was originally
ascribed to the haemolysin, Barclay et al. (1989a) did

comment that the exoproteins might act synergistically.

L. monocytogenes also produces enzymes which protect it from destructive oxidative chemicals found in the phagocytic vacuoles of macrophages. Examples include catalase and superoxide dismutase which inactivate peroxide and the superoxide anion respectively (Bortolussi et al., 1987; Welch, 1987; Benedict, 1990). Furthermore, Galsworthy (1987) suggested the resistance of L. monocytogenes to macrophage lysozyme might be explained by the finding that its peptidoglycan contains glucosamine residues with free amino groups, but did not elucidate further. Possibly the lysozyme is denied access to the target site in the cell by these amino groups.

1.2.2 Listeriosis

Human listeriosis includes a number of clinical manifestations characterised by symptoms which are initially similar to influenza. Neonatal and adult meningitis are the most common of these conditions. In addition, abortion, stillbirth, granulomatosis, biliary atresia, internal and external abscesses, subacute bacterial endocarditis and opportunistic infections of immunosuppressed adults and neonates have been reported (Leighton, 1985; WHO Working Group, 1988; Lacey & Kerr, 1989). Air borne infection can lead to the fatal pneumonic form of listeriosis (Skovgaard, 1989). The effects of listerial infection are therefore very serious.

The WHO Working Group (1988) reviewed recent data on

human listeriosis and concluded that it is predominantly transmitted by non-zoonotic means. While soil may be the source of the organism, transmission to man is primarily from the environment and food surfaces. Other less common forms of transmission include insect vectors, oral and venereal routes (Leighton, 1985; McLauchlin, 1987). Hird (1987) elucidated direct zoonotic transmission of L. monocytogenes from animals to man and cited the example of a farmworker who contracted listeriosis after cleaning a stable contaminated by a sheep infected with the same serotype of L. monocytogenes. Cutaneous listeriosis in vets who attend stillbirths and abortions in cattle and conjunctivitis in poultry workers have also been documented.

A number of species in the genus Listeria have been implicated in the causation of listeriosis. While L. monocytogenes is the principal pathogen, some researchers have commented that L. seeligeri and L. ivanovii can also cause sporadic cases (Schlech, 1989; Schönberg, 1989; Terplan & Steinmeyer, 1989). McLauchlin (1987) stated that four Listeria species have caused infections in animals and man: L. monocytogenes, L. ivanovii, L. innocua and L. seeligeri, but in only three, two and one cases respectively were L. ivanovii, L. innocua and L. seeligeri responsible for human listeriosis. However, 10 % of animal infections are caused by L. ivanovii, mainly ovine abortion.

Over the last 10 to 15 years, the numbers of cases of listeriosis in the UK has risen about 6 to 8 fold

(Adams & Roper, 1990). In 1988 there were 291 cases, with
63 fatalities (Greenwood et al., 1991; Adams & Roper,
1990). Gill (1988) stated that the overall mortality rate
is approximately 30 %, but mortality sometimes rises as
high as 60 % for specific groups including the elderly,
seriously immunocompromised adults, neonates and
developing foeti. Lucas and Levin (1989) reported that
mortality amongst un-treated patients is approximately
70 %. These data indicate that listeriosis is the
commonest cause of death from microbial food poisoning
(Lacey & Kerr, 1989). The latter authors have suggested
that the rise in the number of cases is due to the
increased use of refrigeration in the food industry, as
at low temperatures this psychrotrophic organism can
synthesise its major virulence factor, listeriolysin O,
in increased amounts (Junttila et al., 1988).

Outbreaks of human listeriosis have been associated
with a range of serotypes but as yet no correlation has
been found between serotypes and particular clinical
illnesses (WHO Working Group, 1988). Over a period of
years Ralovich (1984) observed a change in the
distribution of serotypes of L. monocytogenes isolated
from human beings. In the UK during the 1960's serotype
1/2 was isolated most frequently, whilst during the
1970's serotype 4 was predominant (Taylor, 1980). In the
early 1980's the ratio of serotype 4 to serotype 1 was
almost unity (Ralovich, 1984), but in the late 1980's
Donnelly and Briggs (1986) and Schönberg et al. (1989)
reported serotype 4b to cause most human listeriosis. A

more recent study (Greenwood et al., 1991), found 80 % of strains of L. monocytogenes isolated from human infections to be of serotype 4.

McLauchlin et al. (1986) and Kvenberg (1988) both reviewed reports of outbreaks of human listeriosis which occurred in different parts of the world during the last 40 years (data summarised in Table 1.2). As indicated in the table, a number of these outbreaks have been directly associated with the intake of particular foods.

To date information regarding the infectious dose of L. monocytogenes is unclear (WHO Working Group, 1988;

Location	Date	Number of cases			Serotype	Suggested agent of transmission
		Total	Adults	Perinates		
Prague, Czechoslovakia	Aug.-Nov. 1955	41	0	41	1/2	NK
Bremen, Germany	1960-1961	81	NS	NS	NS	NK
Bremen, Germany	1963	20	NS	NS	NS	NK
Halle, Germany	Apr.-Dec. 1966	279	0	279	1/2	NK
Auckland, New Zealand	Apr.-Sep. 1969	20	6	14	NS	NK
Greenville, US	Mar.-Oct. 1975	6	0	6	4b	NK
Anjou, France	Jan.-Jun. 1976	162	36	126	4b	NK
Perth, Australia	Jan.-Mar. 1978	4	0	4	NS	NK
Perth, Australia	Jan.-Mar. 1979	6	0	6	NS	NK
Auckland, New Zealand	Jan.-Jun. 1980	21	3	18	1/2a	Seafood?
Nova Scotia, Canada	Apr.-Sep. 1981	41	7	34	4b	Coleslaw
Houston, US	May -Jul. 1983	10	0	10	1/2b	NK
Massachusetts, US	Jun.-Aug. 1983	49	42	7	4b	Milk
Lausanne, Switzerland	Jan.1983-Mar.1984	25	14	11	4b	NK
Los Angeles, US	1985	86	28	58	4b	Mexican style cheese
Vaud, Switzerland	1983-1987	NS	NS	NS	NS	Vacherin cheese

Key

NK = not known

NS = not stated

Table 1.2 Summary of data on outbreaks of human listeriosis 1955 to 1987 (modified from McLauchlin et al. (1986) and Kvenberg (1988)).

Al-Ghazali & Al-Azawi, 1990; Johnson et al., 1990; Lund, 1990). Ralovich (1987) stated that for healthy human beings the infectious dose is high although it is obviously related to host susceptibility. However, in the Mexican style cheese outbreak of 1985 it was estimated that the contaminated cheese contained approximately 100 to 1000 L. monocytogenes cells per gram and therefore the oral infective dose may have been as low as 100 bacteria (Lund, 1990).

The incubation period of listeriosis can range from one to several weeks. Furthermore, the carrier state may be triggered to illness by factors such as viral infection (WHO Working Group, 1988). The stages of pathogenesis of listeriosis via the oral route are poorly understood (Jay, 1992) but they include attachment, induction of phagocytosis, multiplication and possibly other mechanisms (Ralovich, 1989).

For attachment L. monocytogenes has cell wall structures which could be specific for host cell receptors on the surface of the intestinal epithelia (Ralovich, 1989; Schlech, 1989). The primary site of invasion depends on the species. For example, in guinea pigs invasion is predominantly in the small intestine, whilst in mice it is the Peyers patches which are invaded.

Berche et al. (1987) observed that when cells were injected into the bloodstream they were readily engulfed by macrophages lining the blood vessels inside which they multiplied. However, not all strains of L. monocytogenes that produce infection by injection can produce infection

when administered orally. Schlech (1989) found that while 'smooth' haemolytic strains of L. monocytogenes result in invasive infection when administered orally, 'rough' haemolytic strains did not cross the epithelium. It has been elucidated that L. monocytogenes grown in unfavourable conditions are 'rough' due to the altered protein composition of their cell wall (Hof and Chatzipanagiotou, 1987), and therefore could lack a proteinaceous structure necessary for adherence and invasion. Furthermore L. monocytogenes 'rough' strains were found to be more susceptible to killing by macrophages. The ability of certain strains of L. monocytogenes to attach and invade the gastrointestinal tract therefore gives a competitive advantage over other potential pathogens.

Gaillard et al. (1987b) suggested two routes by which L. monocytogenes could invade host tissues in an oral infection. L. monocytogenes may first penetrate non phagocytic cells and multiply within them. There is evidence that L. monocytogenes is capable of penetrating different types of epithelial cells, such as conjunctival, nasal and lung and then replicates prior to a macrophagic stage. The alternate route involves entry via specialised absorptive cells of the Peyers patches epithelium, termed M cells by Gaillard et al. (1987b). Using these cells L. monocytogenes crosses the intestinal epithelial barrier without significant replication. The bacteria are promptly engulfed by macrophages intimately associated with the M cells and then replicate.

Both avirulent and virulent L. monocytogenes can invade intestinal cells. As avirulent non haemolytic strains of L. monocytogenes enter cells at the same rate as haemolytic strains, haemolysins probably play no part in invasion (Gaillard et al., 1987a; Ralovich, 1989). Gaillard et al. (1987a; 1987b) suggested avirulent non haemolytic L. monocytogenes remained in the phagocytic vacuoles of intestinal cells whereas the haemolysin, listeriolysin O is the active agent that disrupts cytoplasmic membranes to allow L. monocytogenes to escape the vacuole and replicate in the cytoplasm of the host cells. Cowart & Foster (1985) reported that virulent strains had faster rates of growth than avirulent strains, relating to the greater ability of the pathogenic L. monocytogenes to acquire iron. At a later stage listeriolysin O causes lysis of the infected intestinal cells allowing L. monocytogenes to instigate its escape. The virulent bacteria then infect and multiply mainly within macrophages, in the second and more efficient multiplication stage (Gaillard et al., 1987).

Shortly after contact with macrophages Listeria is phagocytised (Tilney & Tilney, 1993). L. monocytogenes stays within the hosts defensive phagocytic cells for the first 2 to 4 days of infection and must therefore survive the antimicrobial activity of the phagocytic vacuoles. Phagocytic cells kill most microorganisms within minutes of ingestion by delivering potent antibacterial agents into the phagocytic vacuole (Bortolussi et al., 1987). In

the case of _Listeria_, T cells activate macrophages to become highly bactericidal (Cluff & Ziegler, 1987; Galsworthy, 1987). During the first 2 h in the vacuole the viable number of _L. monocytogenes_ decrease, after which the surviving cells replicate (Bortolussi _et al._, 1987).

L. monocytogenes is later released from the phagocytic vacuole into the cytoplasm of the macrophage by the action of listeriolysin O and phospholipase C (Tilney & Tilney, 1993), and becomes surrounded by a coat of actin filaments within a few hours. The actin filaments reorganise to form a polar tail up to 5 μm long, which promotes intracellular movement and subsequent spread to adjacent macrophages (Kuhn _et al._, 1990; Mounier _et al._, 1990). Approximately 2.5 h after invasion of the macrophage _L. monocytogenes_ begins to migrate around the cytoplasm at speeds proportional to the length of the actin tail. Rates of up to 1 μms^{-1} have been recorded. Migration is always head first, "like a comet through the sky" (Tilney & Tilney, 1993). Any contact with the plasma membrane of the macrophage during migration causes a protuberance to be generated. The membrane folds tightly around the organism and its tail "like a finger in a glove" and produces a pseudopod up to 40 μm long. When this pseudopod makes contact with a neighbouring macrophage the second macrophage phagocytises the pseudopod of the first. The now doubly encapsulated _Listeria_ then escapes by dissolving both membranes, again by the action of haemolysin and phospholipase C. The cycle can be repeated many times without the _Listeria_ leaving

the cytoplasm and therefore avoiding detection by
circulating antibodies (Tilney & Tilney, 1993).

From the intestinal tract the organism enters the
bloodstream from where it can infect other susceptible
tissues including the placenta in pregnant women. Invasion
of tissues could possibly be aided by the extracellular
invasion associated protein, P60 (Jay, 1992). In addition,
it has been observed that listeriolysin O producing
strains can invade certain tissues whereas listeriolysin O
negative strains can not, even though listeriolysin O is
not involved in entry (Kuhn & Goebel, 1989).

Berche et al. (1987) discussed pathogenesis with
respect to the action of haemolysins and the acquisition
of iron. In iron deprived environments there is very rapid
phagocytosis of invasive bacteria which stimulates the
secretion of listeriolysin O. Internalisation of bacteria
within phagocytic vacuoles favours accumulation of
listeriolysin O which, in turn, leads to the disruption of
intracellular membranes. Disruption of iron enriched
lysosomal vesicles makes the sequestered iron available
for bacterial growth, ultimately stimulating rapid
bacterial replication. However, among its range of defence
mechanisms the host can reduce the availability of iron to
a pathogen by increasing serum transferrin levels (Cowart,
1987). The interplay between haemolysin and the immune
system therefore appears to be a major factor in
determining the outcome of infection with virulent
L. monocytogenes (Cluff & Ziegler, 1987).

Other host defences include the gastric barrier of

the stomach. The ability of L. monocytogenes to withstand a pH shock is therefore important in its survival prior to ingestion by macrophages (Kroll & Patchett, 1992). Additionally, the protective effect of the normal intestinal microflora towards pathogens termed colonisation resistance, may play a significant role (Fuller, 1989). Other species of mammals have additional defences, for example, rabbit blood contains a specific anti listerial agent, called ß lysin (Cowart & Foster, 1985).

1.2.3 Introduction of L. monocytogenes into the food chain

It was suggested 50 years ago that L. monocytogenes could be transmitted to humans through foods (Burn, 1936).

The number of reported cases of listeriosis in the UK has generally increased every year since 1967 (McLauchlin, 1987), however, since 1988 the annual reported cases have dropped to about 130 cases annually (Lacey, 1993). The increase in numbers of listeriosis (up to 1988), is partly due to greater surveillance efforts, changes in agricultural practices, such as the use of the 'big bale' type of silage production, may be a major cause. Listeriosis has been called the 'silage disease', as the bacterium is able to multiply in poorly prepared silage when the pH remains too high. The subsequent feeding of such silage to farm animals introduces Listeria into the food chain (McLauchlin, 1987; Lund, 1990). The WHO Working Group (1988) commented on the link between faecal

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excretion of L. monocytogenes and the condition of silage fed to cows. Skovgaard (1989) reported that in Denmark 67 % of cattle excrete Listeria spp. (53 % L. monocytogenes) and their feed contained 82 % Listeria spp. (62 % L. monocytogenes), indicating a relationship between Listeria spp. ingested in feed and excreted Listeria spp. In contrast to the other authors, however, Skovgaard (1989) believes that the importance of silage has been overstressed. He argues that ruminants feed on vegetables of various kinds and all vegetation can harbour Listeria spp. which multiply during natural decay to a greater extent than in silage.

Whatever the original source, it would be expected that the greater the number of Listeria excreted to the environment, the greater number reintroduced into dairy cows or other meat animals, leading to further contamination. Modern farming practices such as the use of antibiotics as growth promoters, the separation of young animals from their mothers prior to the acquisition of the normal intestinal flora and the stress of battery housing all decrease the resistance of farm animals to colonisation by intestinal pathogens (Fuller, 1989). Glass & Doyle (1989) reported that 3 % of cattle in the US are now carriers of L. monocytogenes.

The increase in listeriosis and diseases caused by other human intestinal pathogens also coincides with the use of untreated sewage sludge and animal slurry as fertiliser on agricultural land. L. monocytogenes is sometimes present in high numbers in sewage sludge and

sewage plant effluents (Lund, 1990), and is able to survive for at least eight weeks when sprayed onto agricultural land (McLauchlin, 1987). Al-Ghazali and Al-Azawi (1990) reported that when L. monocytogenes contaminated sewage sludge cake was used as a fertiliser, 10 % of alfalfa (lucerne) plants were infected at harvest time. Furthermore, Beuchat et al. (1990) have argued that once contamination has occurred, L. monocytogenes can survive on vegetation for 10 to 12 years and can reach populations of 10^5 to 10^7 colony forming units (CFU) per g.

Due to modern farming practices Listeria spp. are now considered to be self perpetuating in agriculture. Figure (Fig.) 1.1 summarises the environmental cycle of L. monocytogenes. Listeria is primarily transmitted to man through foodstuffs contaminated during production and processing (WHO Working Group, 1988; Skovgaard, 1989). L. monocytogenes should be considered to be present on vegetation treated with silage or sewage sludge fertiliser and on meats contaminated with faecal matter in the abattoir (WHO Working Group, 1988; Skovgaard, 1989). All raw milk should be considered to harbour L. monocytogenes of faecal origin at a concentration of less than one cell per cm^3 (Skovgaard, 1989), while milk from Listeria infected cows with mastitis may contain 10^3 cells per cm^3 (WHO Working Group, 1988). Bulk milk tanks take up to 6 h to return to 4 °C after each milking, allowing ample time for any L. monocytogenes in the milk to multiply (Donnelly & Briggs, 1986).
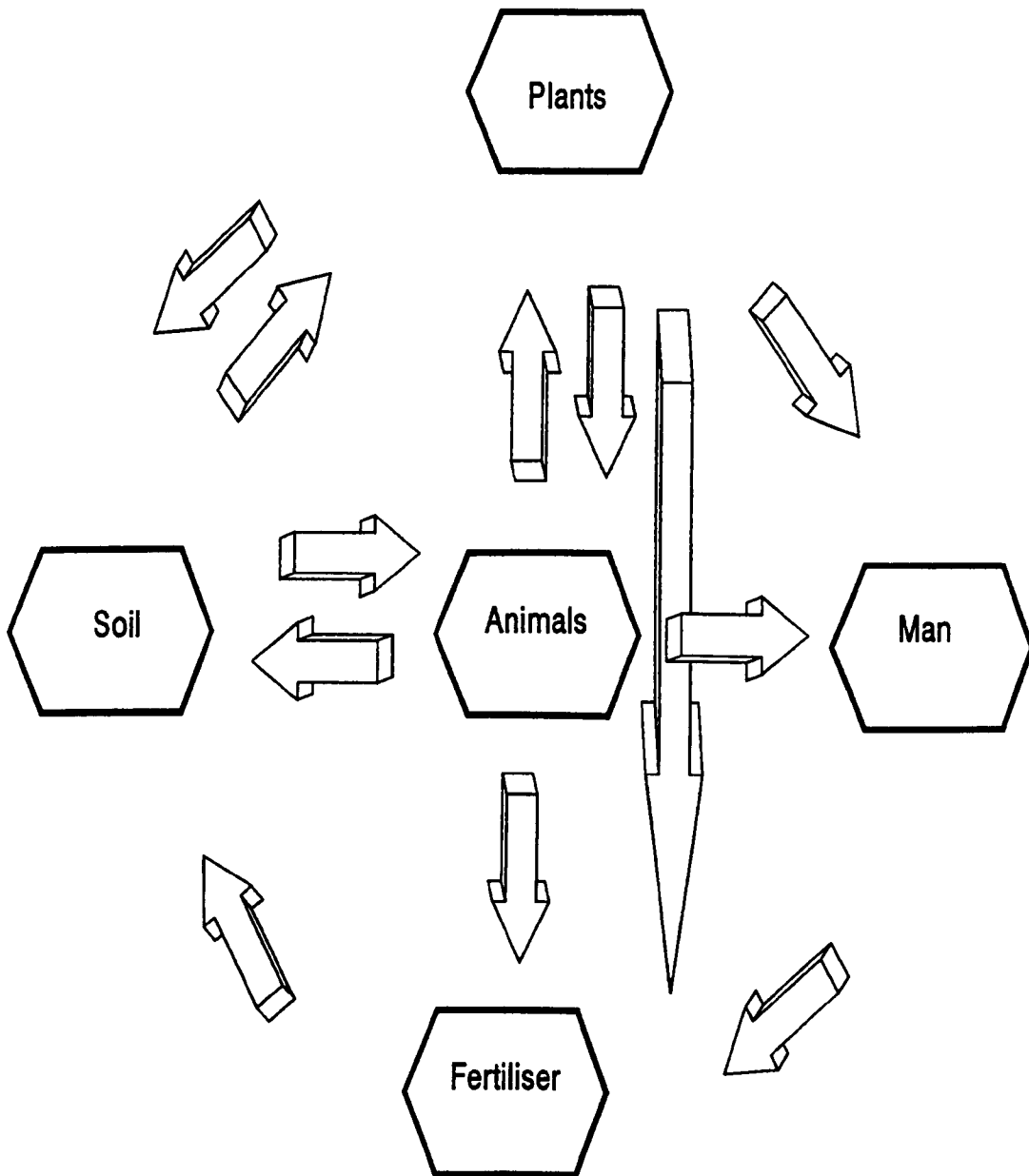
Fig.1.1 Environmental cycle of <u>L. monocytogenes</u>. Arrows indicate transfer of <u>L. moncytogenes</u> between environmental niches. Transfers occur by death and decay of plant and animals material, animal excretion, use of contaminated fertilisers, and ingestion of contaminated foodstuffs.

Contamination of foodstuffs with _Listeria_ can also occur during processing of raw food products, and subsequent distribution and handling by retailers and consumers. In the processing plant organisms may be transferred from food contact surfaces (such as conveyor belts, knives and cutting surfaces), water supplies, insects, rodents and humans (WHO Working Group, 1988). Approximately 3% of humans are asymptomatic carriers of _Listeria_ (Lund 1990). In addition humans may convey _Listeria_ via mud on shoes etc. _L. monocytogenes_ can also be transferred directly between food surfaces, for example, beef tissue slices (Dickson, 1990a). In addition, failures in the pasteurisation process (for example, through pin-holes in the heat exchanger) may allow _Listeria_ to contaminate food during processing.

Skovgaard (1989) discussed data collected by the American Meat Institute on 41 meat plants: 29% of floors, 37% of drains, 20% of food contact surfaces and 5% of walls and ceilings in the post heat processing areas were found to be contaminated with _Listeria_. Post processing contamination is a very important factor in cheese production too. Cheeses made with pasteurised milk are as frequently contaminated with _L. monocytogenes_ as cheeses made from unpasteurised milk (WHO Working Group, 1988). _L. monocytogenes_ thrives in cool room conditions of damp and cold (Venables, 1989). Condensation can develop on such cool room ceilings and fall onto exposed products, for example ricotta cheese packed into perforated containers to drain.

Wehr (1987) has commented that Listeria frequently remains in processing plants despite industry's best efforts to eliminate it through vigorous sanitation efforts. Research is being undertaken into controlling L. monocytogenes by biocides (Rossmoore & Drenzek, 1990). However caution is required in selecting a disinfectant for L. monocytogenes as this organism is difficult to inactivate when on surfaces particularly in the presence of organic matter (Best et al., 1990).

Lund (1990) reviewed extensive surveys of the incidence of L. monocytogenes in different types of foods (see Table 1.3 for summary). The problem of listeriosis is international. For example, L. monocytogenes has been found in frozen seafood products from nine different countries (Fuchs & Surendran, 1989).

In a variety of meats L. monocytogenes 1/2a was observed to be predominant serotype (86 of 126 Listeria isolates from 265 food samples). Other serotypes detected included 1/2b, 1/2c, 3a, 4a, and 4d. However serotype 4b, which currently causes 2/3 of human disease was only present in a few samples (4 out of 126) (Schönberg et al., 1989). Of 89 cheese samples, 9 were found to contain L. monocytogenes, 5 were of serotype 1/2a and 4 of serotype 1/2b (Schönberg et al., 1989).

1.2.4 Resistance of L. monocytogenes to food processing treatments

L. monocytogenes is exceptionally resistant to the

Food	Total no. of samples	No. of positive samples	% of positive samples	CFU per gram	Reference
ground beef	22	17	77		2
raw beef	29	11	38	$<10^2-10^3$	3
ground pork	19	18	95		2
raw pork	33	12	36	$<10^2-10^6$	3
raw pork sausages	25	13	52		1
raw minced meat			up to 30		4
cooked, cured/smoked meat	29	2	7		1
salami and continental sausages	67	11	16		1
fermented sausages	30	6	20, up to 20		2, 4
chilled patés	1774	180	10		5
patés	73	37	51	$<20->10^5$	9
raw chicken	100	60	60		1
raw poultry	14	6	15-18	$<10^2$	3, 4
raw milk	137	6	4, up to 5	$<10^2$	3, 4
milk - pasteurised	41, 14	0, 0			3, 2
Cheese - camembert/brie					
made from raw milk	18	10	55	10^3-10^6	3
made from pasteurised milk	51	0			3
soft cheese	222	23	10	$<10^2-10^5$	1,8
soft ripened cheese			1-10	10^4-10^7	4
ice cream	394	1	0.25, 0-5.5	1-15	2, 4
shrimps, cockles	40	0			1
frozen seafood products	57		26	10^4-10^7	6
pre-packed salads	60	4	7		1
fresh cut vegetables (salads)	25	11	44	10^2	3
lettuce	50	0			2
celery	30	0			2
radishes	10	0			2
tomatoes	20	0			2
lettuce	92	0			7
cabbage	92	1	1		7
radishes	132	19	14		7
potatoes	132	28	21		7
From retail premises					
pre-cooked ready-to-eat poultry	527	63	12		1
chilled meals, mainly poultry	74	13	18		1
From cook-chill catering units					
main course items	627	10	2		1
desserts	73	0			1

References

1 = Gilbert et al. (1989) 2 = Farber et al. (1989a)
3 = Beckers et al. (1989) 4 = WHO Working Group (1988)
5 = Department of Health (1989) 6 = Weagent et al. (1988)
7 = Heisick et al. (1989a) 8 = Pini & Gilbert (1988) 9 = Morris & Ribeiro (1989).

Table 1.3 Incidence of L. monocytogenes in a variety of foodstuffs (modified from Lund, 1990).

physical factors that normally limit the numbers and
incidence of microorganisms in foods.

It is psychrotrophic and in the laboratory is capable
of growth at refrigeration temperatures as low as -0.4 °C
as long as the medium remains unfrozen (Walker et al.,
1990). Junttila et al. (1988) suggested that this
characteristic is due to its possession of enzymes which
are active at low temperatures, a cold resistant sugar
transport system and high concentrations of unsaturated
fatty acids in the membrane which protect the cells from
freezing. L. monocytogenes can adapt itself to cold
temperatures, reducing lag times when inoculated from a
chilled medium into fresh chilled medium (Walker et al.,
1990). It can also survive frozen storage (Olsen et al.,
1988), although Johnson et al. (1990) reported 82 % of
cells were injured. In contrast, Golden et al. (1988b) and
Farber (1989b) found that freezing had no detrimental
effect on L. monocytogenes.

Although it is widely known that L. monocytogenes is
more heat resistant than other non-sporing foodborne
pathogens, the extent of its resistance is still
controversial (Golden et al., 1988b; Mackey & Bratchell,
1989; Lemaire et al., 1989). There are many reports of its
unusual resistance when heated in meat or poultry products
(see Mackey & Bratchell (1989) for review). Heating
chicken breasts to an internal temperature of 71.1°C
caused a reduction in the population of L. monocytogenes
of only approximately 1.3 to 2.3 logs (Harrison &
Carpenter, 1989). However other researchers, including

Mackey & Bratchell (1989) themselves, have not observed unusual heat resistance in meat and poultry products, and they concluded that reports of such resistance probably derive from uneven heating. Farber (1989a) also reported that some meats may not have been heated adequately for L. monocytogenes to be inactivated even if present in low numbers.

The resistance of L. monocytogenes to pasteurisation in milk has also been controversial. Farber (1989a) reviewed this field and commented that while early workers suggested L. monocytogenes had unusual resistance to pasteurisation, later workers disagreed indicating that experimental technique in early experiments was at fault. It has been suggested that as L. monocytogenes is an intracellular pathogen it could survive within bovine leukocytes due to the protection these would afford. However, Mackey and Bratchell (1989) reported that there is no evidence for encapsulation of L. monocytogenes by bovine leukocytes.

L. monocytogenes is able to adapt after a heat shock to resist further heat treatment. Farber (1989a) has stated that this effect could be significant for bulk foods which are heated slowly, or other foods which receive a marginal heat treatment.

L. monocytogenes is one of very few foodborne pathogens capable of surviving below a water activity of 0.93. Solutes which reduce the water activity have been used historically for preserving foods, though the presence of high concentrations of salt and sugar has

little detrimental effect on L. monocytogenes. It has been reported that L. monocytogenes can grow in 13 to 14 % salt solution at decreased temperatures (Farber et al., 1992) and can survive 20 % salt solution (Farber, 1989b). It will also grow in broth containing 39.4 % sucrose (Farber et al., 1992).

L. monocytogenes can survive extremes of pH, again a factor that has traditionally been used to control microbial contamination. Its growth range is normally given as being approximately 5.5 to 9.6, though below a pH of 5.5 L. monocytogenes is reputed to survive but not multiply (Wehr, 1987; George et al, 1988). Kroll & Patchett (1992) have shown that L. monocytogenes can adapt to tolerate lower pH's when grown initially in low pH media. This could result in cells from low pH food being able to tolerate stomach acid to a greater extent than normal, effectively reducing the infectious dose.

L. monocytogenes is capable of survival in lactic mixed cultures (Schaak & Marth, 1988a, 1988b). Because of its psychrotrophic nature L. monocytogenes is more capable of competing at low temperatures, but it has been observed to survive all mesophilic lactic fermentations traditionally used to decrease spoilage of foodstuffs (Schaak & Marth, 1988b). Carminati et al. (1988) reported inhibition not inactivation of L. monocytogenes with all starter cultures examined. Schaak & Marth (1988b) commented that antagonism towards pathogens could not be predicted by the rate of acid production alone in lactic culture as additional factors contributed including the

antibiotics nisin and lactostreptins, volatile compounds and hydrogen peroxide. Hydrogen peroxide production, however, can also lead to increased growth of L. monocytogenes by decreasing numbers of competitive microflora. Harris et al. (1989) suggested L. monocytogenes overcomes bacteriocins by proteolytic degradation or by outgrowth of a resistant mutant population. Furthermore, it has been shown that casein can exert a protective effect for S. typhimurium in acid dairy products (Schaak & Marth, 1988a). The same could be possible for L. monocytogenes. Other bactericidal factors in milk, such as lactoperoxidase, lysozyme and agglutinines, decreased the growth of L. monocytogenes during the first two days of storage (Northolt et al., 1988). However, L. monocytogenes is able to survive storage in lactic products for long periods of time. For example, it has been shown to survive in cold pack cheese for 434 days, though it will not grow in any cheese below a pH of 5.45 (Ryser & Marth, 1988).

L. monocytogenes can tolerate the presence of many antimicrobial food additives and curing agents currently in use (Lammerding & Doyle, 1990), such as nitrates (Doyle, 1988; Lacey & Kerr, 1989) and sodium chloride (Olsen et al., 1988). Antimicrobial products from microbial metabolism, for example lactic acid, are now being considered for use in the food industry as decontaminants (Farber et al., 1989b), as is the direct addition of bacteriocins, for example nisin, to act as preservatives (Farber, 1992).

It has been reported that some strains of
L. monocytogenes isolated from meat products have
non-plasmid mediated resistance to antibiotics (Barbuti et
al., 1992). This could indicate that these strains have a
human or cattle origin. The genetic transfer of antibiotic
resistance between strains of L. monocytogenes has been
observed to be difficult in vitro (Lucas & Levin, 1989),
but sublethal stress damaging the cell wall may allow this
to occur in vivo.

Food packaging can also affect the growth of
contaminating L. monocytogenes. Kvenberg (1988) and
Skovgaard (1989) reported that growth increases under
decreased oxygen tension and with supplementation of
carbon dioxide.

1.3 Current food safety legislation in the UK

Since the Food Safety Act 1990 (which came into operation
in the UK on January 1st. 1991), the food industry in the
UK has had to control the standard of hygiene of foods in
ways and according to regulations which differ
significantly from earlier legislation. The Act applies
throughout the UK with similar but separate legislation
for Northern Ireland. The main features of the Act may be
summarised as follows (modified from Davies (1992):

1. The new law applies to everyone working in the
   production, processing, storage, distribution or sale
   of food. The law applies to all food businesses,

including the self-employed. All food businesses must register their premises (including such vehicles as ice-cream vans) with their local district council. Registration is free but not an endorsement of hygiene practices being within the law.

2. The law states that it is a criminal offence to render food injurious to health, or to sell such food or keep such food for eventual sale. The law specifies offences regarding contaminated food and food products which are not of the quality demanded by the purchaser. It is an offence to make misleading or false claims about food items. The law also specifies regulations about hygiene practices (such as storage temperature specifications for perishable foods in refrigerators) and conditions of hygiene. Under the term "food" the Act covers many items including food ingredients, drinks, slimming aids taken orally and dietary supplements. It does not cover medicines (which are regulated by other laws) or animal feedstuffs.

3. The new law is enforced by local authority officers such as Environmental Health Officers and Trading Standards Officers, who also offer advice on the best codes of practice. Local authority officers have new powers to enter premises to inspect food hygiene. They may seize food which is deemed to be unfit for human consumption and seek enforcement of the law through the courts. Authorised officers now have more extensive powers to require improvements to unhygienic premises and to close premises which pose a public health risk.

Closed premises may open only after
re-inspection and certification that they no longer
pose a health risk.

4. The law also requires everyone who handles food which
   is to be sold to have undergone recognised hygiene
   training.

The new Food Safety Act, based on the European
Community Directive number 397 in 1989, was a major
improvement on previous legislation such as the UK Food
Act 1984. Although this former Act could allow prosecution
of suppliers of food unfit for human consumption, it was
vague and broad based (Lacey & Kerr, 1989). In addition to
the new food laws, the food industry now has to comply
with legislation such as Control of Substances Hazardous
to Health (COSHH Assessments, 1988) and other Health and
Safety regulations, for example, the Health and Safety at
Work Act (Ritson, 1983). With regards to product safety
there is strict liability; anyone who suffers damage due
to a defective product can now recover damages without
having to prove negligence (Tuley, 1991).

Although food safety has recently become much more
enforceable, the food industry in general has been
attempting to eliminate the problem of microbial
contamination of foods for a number of years. Lund (1990)
disclosed that in 1988 guidelines were prepared by members
of the UK Dairy Trade Federation for the improvement of
hygienic practices in the production of soft and fresh
cheeses. In addition guidelines for production,

distribution and retail handling of chilled foods were produced by the Chilled Food Association, and the Department of Health produced up to date guidelines for cook-chill and cook-freeze systems.

Furthermore, the cost of recalls in terms of profit and reputation puts pressure onto the food industry to ensure product safety. For example, the salmonellosis outbreak in Cumbria, England in 1985 (76 recorded cases and one infant death) which resulted from the consumption of contaminated infant dried milk, led to a profit reduction of more than £22 million for the company concerned and the closure of one of the two production units, with the loss of more than 100 jobs (Waites & Arbuthnott, 1991). Unsafe food has to be withdrawn from sale and destroyed as adverse publicity can lead to further economic losses. For example, sales of corned beef after the 1964 typhoid outbreak in Aberdeen, Scotland were not restored to pre-outbreak levels for 20 years (Waites & Arbuthnott, 1991).

Campylobacter, which has now become the most commonly reported cause of foodborne infection in England and Wales (Waites & Arbuthnott, 1991), was reported to have cost nearly £9 million in 1989. Direct costs were in health care, laboratory and environmental health investigations, while indirect costs involved mainly lost productive output. The cost of pain and suffering, however, could not be evaluated (Skirrow, 1991).

1.4 Review of current methods used to monitor the microbiological quality of foods

1.4.1 Strategies for safety assurance

It is over fifty years since it was first suggested that
L. monocytogenes could be transmitted through foods (Burn,
1936) and over twenty years since it was postulated that
the growing use of refrigeration could lead to an increase
in the incidence of listeriosis (Kramer & Jones, 1969).
However, the development of techniques to eliminate
L. monocytogenes and other food poisoning organisms from
the production environment and of detection methods to
monitor pathogens in foodstuffs has been slow. For economy
and to maintain organoleptic quality, the food industry
has traditionally used automatic and high speed equipment
and minimum processing times and temperatures.
Formulations were made to decrease preparation time by the
consumer. Until recently food safety has not been a
priority.

The principles of preventative microbiological safety
assurance were formulated as early as 1935. The essential
elements of these became known as the Wilson Triad
(Mossel, 1989), namely:

1. Elimination of unwanted organisms by i) restricting
colonisation of raw materials to a minimum and ii)
enhancing microbial lethality by processing to the
highest level compatible with preservation of nutritive
value and organoleptic quality.

2. Avoidance of recontamination of treated commodities

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by i) processing after hermetic packaging or ii) aseptic packaging.

3. Distribution and storage of the commodities under conditions i) arresting the proliferation of viable organisms that survive processing and ii) delaying recontamination.

Independently Bauman (1974) introduced a new safety assurance strategy termed Hazard Analysis / Critical Control Points (HACCP). This involved control from 'source to kitchen', namely from production areas, through processing and packaging, to consumption. To ensure end products meet microbiological safety criteria, "Hazard Analysis" involves identification of sensitive ingredients and processing steps as well as relevant human factors, such as consumer abuse of products. Ingredients are assigned to categories in descending order of risk. For example, meats, which can potentially carry pathogens, are classified as hazardous whilst sugar, salt and similar dry commodities are regarded as safe. In addition predictions are made about consumer treatment of food and tolerance ranges determined. The "Critical Control Points" are those processing stages where any loss of control would result in unacceptable product safety risk. There are two types of critical control point:

1. Points at which microorganisms can be eliminated. Failure to maintain and monitor the process within certain limits may result in a microbiologically unsafe food. Pasteurisation is an example of such a critical

control point.

2. Points where control is important to minimise contamination and growth. Temperature control during storage is an example of this type of critical control point.

HACCP states that all correctable hazards should be eliminated and then a system of control should be established for any hazardous process that remains part of the production line. Such a system is easily established by determining specifications for ingredients, manufacturing, packaging, product testing and so on. Any deviations are reported to and controlled by a data capture and analysis section, which decides the remedial action necessary.

The Wilson Triad and HACCP safety approaches have been practised since their development by the Dairy Industry. HACCP was later suggested for general adoption by the food industry (Mossel, 1983; Heeschen, 1987). Coleman (1986) has described a HACCP regimen specifically for Listeria. This acknowledges the fact that Listeria is a very resistant ubiquitous organism.

BS5750 is a standard recently introduced to the food industry that requires a high degree of process reliability from the acquisition of raw materials to the release of finished product (bioMérieux, 1991). It includes as a major component the traditional methodology of microbiological quality control and assurance (Ratcliffe, 1993) and certification of compliance is given

to any manufacturing or service sector organisation which
has proven through audit that it has suitable quality
management systems (Davies, 1993). In addition to
traditional microbiological safety regimens, this new
standard stresses the importance of overall management of
quality, for example in ensuring the ability to locate
unsafe food items that have to be recalled after
distribution (Ratcliffe, 1993).

1.4.2 Development of new microbiological procedures

Recent legislation together with the development of safety
assurance strategies have led to a change in the attitude
of the food industry and policies are now based on safety
rather than economy. Roberts & Jarvis (1983) suggested
three possible outcomes of this change. Firstly, in order
to speed up quality control, traditional microbiological
methods could be replaced with automated versions.
Secondly, monitoring systems based on microbial activity
rather than detailed identification of microorganisms
could be developed. Thirdly, use could be made of
predictive microbiology to assess likely microbial growth
responses during food storage. All three approaches have
been explored.

Methods have been developed to decrease the time and
labour intensity of a number of microbiological
techniques. Fung (1992) described some of the available
equipment: The gravimetric diluter automatically prepares
accurate dilutions of samples, the spiral plating system
distributes a thin layer of sample in a spiral manner at a

decreasing rate from the centre to the edge of an agar plate resulting in continuous dilution, while the laser colony counter detects the presence of colonies by a light sensor.

Among methods available to detect microbial activity are impedance microbiology (described in section 1.5) and bioluminescence. Sharpe (1986) elucidated the latter technique for the determination of microbial loads. In the presence of adenosine triphosphate (ATP), the firefly luciferin - luciferase system emits light which can be monitored by a luminometer. The amount of light produced by the system is proportional to the amount of ATP, and, as the amount of ATP per bacterial cell is generally constant, this relates to the number of bacteria (Jay, 1992). Unfortunately the use of this method is restricted by the difficulty of separating bacterial ATP from ATP in foodstuffs (Sharpe, 1986). In addition bioluminescence is not able to differentiate contamination by particular organisms (McAthey & Bointon, 1990).

Predictive microbiology involves extrapolation from observations made in model systems in order to estimate the rate and type of microbial growth in more complex environments such as food products (Roberts & Jarvis, 1983). McMeekin & Olley (1986) listed many examples of the application of this technology, such as the prediction of the shelf life of fish stored at different temperatures in modified atmospheres including vacuum packing.

1.4.3 Traditional methods used in the detection of
L. monocytogenes

There is no standard method for the detection of
L. monocytogenes (Skjerve et al., 1990). A number of
different strategies have been described over many
decades. Early attempts included the Henry (1933) method
which involved basal illumination at a 45° angle of growth
on a non-blood agar medium. Under such conditions
L. monocytogenes colonies have a distinctive appearance
(Donnelly, 1988). Gray et al. (1948) developed a method of
enrichment for Listeria which took advantage of its
psychrotrophic nature. Specimens under analysis are
refrigerated in a non-selective media, such as blood
containing tryptose soy broth. However, this procedure can
take as long as six months (Donnelly et al., 1988).

Chemicals as selective agents for L. monocytogenes
were first recommended by Schoer (1944), who suggested the
use of potassium tellurite as an inhibitor of Gram
negative organisms. Since this time many different
Listeria selective media have been formulated. Those of
primary importance include the medium of McBride and
Girard (1960) containing lithium chloride and
phenylethanol. Beerens and Tahon-Castel (1966) and
Ralovich et al. (1970; 1971) developed the use of
nalidixic acid and acriflavine respectively to inhibit
non-listerial microbes. Much research has concentrated on
enhancing the selectivity of media. Many individual and
combinations of selective agents have been evaluated for
incorporation into new direct plating media and

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traditional enrichment broth and selective agar. See, for example, Kramer & Jones (1969), Dominguez et al. (1984), Doyle & Schoeni (1986), Lee and McClain (1986), Buchanan et al. (1987), Bannerman & Bille (1988), Loessner et al. (1988), van Netten et al. (1988), Curtis et al. (1989a), Heisick et al., (1989b) and Golden et al. (1988a; 1990).

Colour change indicators of the growth of Listeria spp. have also been used in selective media (Eley, 1990). For example, McBride and Girard (1960) incorporated blood into their medium enabling identification of L. monocytogenes by the appearance of ß-haemolysis around the colonies. Listeria spp. are consistently aesculinase positive, and, in the presence of aesculin and iron salts, cause blackening of agar. While many species of enterococci can grow on Listeria selective media and are also aesculinase positive, the addition of mannitol, which is invariably utilised by Enterococcus spp. but not by Listeria spp., can be used to differentiate these two genera (van Netten et al., 1989).

The suitability of a particular L. monocytogenes plate counting procedure is influenced both by the purpose of the analysis and the type of food being analysed. Foods with high loads of contaminating microflora require highly selective media (Brackett & Beuchat, 1989). Furthermore the number of target organisms likely to be present in a foodstuff is important as low levels often necessitate selective enrichment to allow these cells to multiply to a concentration detectable by either a traditional plate method or one of the newer rapid methods (Yousef et

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al., 1988). Little information is known about the
behaviour of L. monocytogenes in enrichment broths,
however, one cell of L. monocytogenes has been reported to
yield greater than 5×10^6 CFU cm^{-3} after 12 to 16 h
(Fernandez-Garayzabal & Genigeorgis, 1990). Unfortunately
enrichment procedures preclude microbial enumeration under
normal use (Yousef et al., 1988) and increase assay times
(Slade & Collins-Thompson, 1988). Some selective media,
termed direct plating media, are inoculated directly from
foodstuffs (that is, without enrichment) and are used for
enumeration. One of the most efficient for recovering
Listeria is lithium chloride phenylethanol moxalactam
(LPM) agar (Cassiday et al., 1989; Lammerding & Doyle,
1989; Loessner et al., 1988). However Fernandez-Garayzabal
& Genigeorgis (1990) commented that plates inoculated with
food samples are generally overgrown by background
microflora and stated that direct plating is unsuccessful
in most cases.

The United States Department of Agriculture (USDA)
method, developed by McClain & Lee (1988), is an example
of a two stage detection assay involving a selective
enrichment stage preceding plating onto LPM agar (for
schematic diagram see Pusch, 1989). Many other detection
regimens have been developed. For example Szemérdi (1990)
and Yu & Fung (1992) utilised the combination of a
selective semi-solid medium in a U shaped glass tube and
the motility of Listeria spp. If a foodstuff contaminated
with L. monocytogenes was inoculated into one side of the
U tube, the motile organism travelled through the

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semi-solid selective medium and after incubation a sample could be taken from the other side of the tube for confirmatory assays. Yu and Fung (1992) incorporated the enzyme oxyrase into their semi-solid selective medium. This was found to increase the growth rate of L. monocytogenes allowing more rapid detection. Yu and Fung (1992) showed the U tube method to be as sensitive as the USDA method with a shorter detection time. Furthermore, it was able to detect 11 samples contaminated with L. monocytogenes which had been missed using a deoxyribonucleic acid (DNA) hybridisation procedure (see 1.4.4).

A number of difficulties have been experienced with selective methods. These limitations include lack of sensitivity (Buchanan et al., 1987; Loessner et al., 1988; van Netten et al., 1988; Golden et al., 1988a; 1990). In addition, some agents used in Listeria selective media have been reported to be inhibitory to L. monocytogenes (Leighton, 1985; Loessner et al., 1988; van Netten et al., 1988; Curtis et al., 1989b). Northolt et al. (1988) found 86% of L. monocytogenes isolates could not grow on trypaflavine-nalidixic acid agar as described by Ralovich et al. (1970) and Kampelmacher and van Noorle Jansen (1980). They reported that even after replating onto nutrient medium the bacteria appeared to remain in a "lag" phase. The use of selective agents could therefore exaggerate the difficulty of recovering sublethally injured L. monocytogenes from foodstuffs.

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1.4.4 Rapid methods used in the detection of
L. monocytogenes

Using traditional selective techniques the isolation and subsequent identification of L. monocytogenes takes a minimum of ten days (McLauchlin & Pini, 1989). Recent concern about foodborne listeriosis has provided the impetus for the development of more rapid diagnostic tests. The aim of these new methods is to increase the capacity, reproducibility and reliability of screening food products for L. monocytogenes. Techniques that have been described include flow cytometry (Donnelly et al., 1988; Eley, 1990), immunological assays (Farber et al., 1988; Mattingly et al., 1988; McLauchlin & Pini, 1989; Jay, 1992), nucleic acid hybridisation (Datta et al., 1988; Klinger et al., 1988; Datta & Wentz, 1989) and the polymerase chain reaction (PCR) (Bessesen et al., 1990; Wernars et al., 1991).

Flow cytometry is a procedure that permits simultaneous enumeration and identification of cells as they travel through a laser beam (Donnelly et al., 1988). Specificity is achieved by the use of DNA-specific fluorescent dyes or fluorescent monoclonal antibodies (Eley, 1990). Under ideal conditions individual species exhibit a characteristic "signature" (that is pattern of light scatter and fluorescence) which is related to morphology, cell size and nucleic acid content (Donnelly et al., 1988). However, the limits of detection of Listeria in milk have been reported to be only

2×10^6 CFU cm^{-3}. This method is clearly insufficiently sensitive for use in foods. Furthermore, the equipment is expensive and complicated to use (Eley, 1990), and the technique suffers a high rate of false positives (Donnelly et al., 1988). In addition, identification based partly on the shape of the bacterium may be hindered by cell wall targeting antibiotics which are used in some enrichment media.

A variety of types of immunoassay have been employed in the detection of L. monocytogenes. Immunoassays involve the attachment of an antibody specific for the target organism to a radioactive element (radioimmunoassay or RIA) (Jay, 1992), to an enzyme (enzyme immunoassay or EIA) (Farber et al., 1988), to an enzyme that can produce a colour change (enzyme linked immuno sorbent assay or ELISA) (Mattingly et al., 1988), or to a dye detectable by fluorescence microscopy (for example the direct immuno fluorescent test or DIFT) (McLauchlin & Pini, 1989). After interaction with the sample under test, any unattached antibody is washed away leaving the antibody conjugate attached to the target organism. Problems of cross reactivity have been noted in certain of these immunoassays (Donnelly et al., 1988; Heisick et al., 1989b; McLauchlin & Pini, 1989; Skjerve et al., 1990). Donnelly et al. (1988) commented that this was caused by non Listeria microorganisms rather than by foodstuffs. The DIFT test, however, had difficulty in detecting L. monocytogenes from cheeses with rinds (McLauchlin & Pini, 1989). Eley (1990) reported a number of problems

associated with ELISA tests for L. monocytogenes. Although this method does not need pure cultures, typically a two day enrichment is recommended followed by the two hour test. Furthermore, the procedure requires a time consuming antigen extraction stage and is only specific to genus level. Recently Sheridan et al. (1991) described an indirect immunoassay for Listeria that combines a micro colony technique with an immunofluorescence test. A filtrate of a crude food homogenate is incubated for 3 h at 37 °C on Listeria selective agar. Anti Listeria antibodies are applied to the filter and incubation is continued for 30 minutes. A fluorescent antibody conjugate is then added to bind to the first antibody and the filter is examined for presence or absence of fluorescing micro colonies. Unfortunately, enumeration is not possible with this technique as the fluorescence fades too quickly and it lacks sensitivity, the limits of detection being 1×10^5 CFU g^{-1}.

Nucleic acid hybridisation involves the binding of a radioactive ^{32}P ribonucleic acid (RNA) or DNA probe to the complementary DNA of the target organism. Hybridisation is then detected by autoradiography. This technique has been used by a number of workers for the detection of L. monocytogenes, including Klinger et al. (1988) and Datta & Wentz (1989). The probe used by Klinger et al. (1988) was thought to be genus specific, whilst that of Datta and Wentz (1989) hybridised only to L. monocytogenes. Nucleic acid hybridisation requires the use of radioactive isotopes with their associated safety

problems. As an alternative, an enzyme such as alkaline phosphatase or peroxidase can be conjugated to the nucleic acid probe (Jay, 1992) in a spectrophotometric assay. However, some foods have been found to give high spectrophotometric readings even in the absence of L. monocytogenes (Eley, 1990).

Due to the lack of sensitivity of nucleic acid hybridisation assays, with limits of detection being estimated as 1 x 10^5 CFU cm^{-3} (Eley, 1990), techniques have been developed to amplify target nuclear material. One method involves the enzyme Qbeta replicase which amplifies the bound probe target complex after hybridisation by as much as a billion fold in 30 minutes (Eley, 1990). A more widely used procedure, PCR, amplifies DNA fragments after hybridisaton of primer sequence with the target DNA. PCR greatly increases sensitivity, enabling 1 to 10 CFU cm^{-3} to be detected in pure cultures and thus eliminates the need for enrichment (Wernars et al., 1991). Unfortunately many problems have been encountered with the application of the PCR technique to foods. Due to their complex composition extensive purification procedures are required to obtain DNA preparations suitable for amplification. Certain cheese components inhibit the amplification reaction and "clean up" procedures can decrease the concentration of DNA available for the reaction. Wernars et al. (1991) found detection limits depended on the cheese used. With some cheeses 2 x 10^3 CFU L. monocytogenes g^{-1} are required for positive detection, a level of contamination which can

readily be detected by direct plating. In other cheese samples 2×10^8 CFU <u>L. monocytogenes</u> g^{-1} were not detected. Bessesen <u>et al</u>. (1990) described the use of PCR to amplify the listeriolysin O gene of <u>L. monocytogenes</u> and allow differentiation of pathogenic and non pathogenic strains. Once again, however, the technique was not sensitive enough to detect less than 10^3 CFU cm^{-3} in foods. In addition to being insensitive, PCR is labour intensive and uses hazardous chemicals such as ethidium bromide. Furthermore, the DNA from cells killed during food processing can also hybridise to probes and produce false positive results (Wernars <u>et al</u>. 1991).

In summary, most of the currently available "rapid methods" share two major disadvantages, the necessity for a enrichment step at levels of contamination likely to be found in foods and labour intensity. Thus there remains the requirement for a still more rapid assay for <u>L. monocytogenes</u>. Such a diagnostic test would be a great benefit to the food industry as it would permit the early identification of contaminated products, and therefore reduce the need for recall after distribution. A rapid technique would also be beneficial in medical diagnosis and would enable the correct treatment to be administered with minimum delay.

1.5 Review of impedance microbiology

It was observed nearly a century ago that the metabolism of microorganisms brings about changes in their growth medium which can be monitored electrically (Stewart,

1899). However, only in the last decade has this property been exploited commercially as a rapid method for the detection and enumeration of microorganisms (Connolly et al., 1988). The following section is an introduction to such electrical measurement, known as impedance microbiology, the technique which is to be evaluated in this research for the rapid screening of L. monocytogenes.

1.5.1 Theory of impedance microbiology

When two electrodes are placed in an electrolyte and a alternating potential difference is applied between them, an electric current is produced in which ions move towards the electrode of opposite charge. The flow of charge (current) is maintained by ions taking electrons from or transferring electrons to the electrodes (Owens, 1985).

Three monitorable electrical signals, conductance, capacitance and impedance, have been used in the detection of microbial activity (Firstenberg-Eden & Eden, 1984). Conductance (G) (the reciprocal of resistance (R)) is the measure of the flow of electric current through a solution (Firstenberg-Eden & Zindulis, 1984). Its value is determined by the concentration of ions in the fluid and the conductive signal results from changes occurring in solution (Firstenberg-Eden, 1986). Conductance is measured in reciprocal ohms (mhos) or in Siemens units (Firstenberg-Eden & Eden, 1984).

Capacitance (C), measured in Farads or microFarads, results from the accumulation of charged ions in close proximity to the electrodes (Firstenberg-Eden, 1986). Most

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importantly cationic protons move to the cathode and are repelled from the anode causing an "electrical double layer" at the interface between each electrode and the solution which stores electrical energy (Firstenberg-Eden & Zindulis, 1984; Owens, 1985). The capacitive signal results from changes in this double layer (Firstenberg-Eden & Zindulis, 1984; Firstenberg-Eden, 1986).

Impedance (Z) is a combination of both conductive and capacitive signals (Firstenberg-Eden & Zindulis, 1984). It has been reported, however, that monitoring instruments do not measure complex impedance but some function of it (Firstenberg-Eden & Zindulis, 1984; Hadley & Yajko, 1985). Impedance is representative of the impedance of the bulk fluid and the polarisation impedance at the interface of the solution and the electrodes. Between 400 Hz and 25 KHz the bulk fluid impedance is mainly resistive (that is, is the reciprocal of conductance) (Hadley & Yajko, 1985), whereas the polarisation impedance has both resistive and capacitive components which are frequency interdependent (Firstenberg-Eden & Zindulis, 1984). Fig. 1.2 is a schematic diagram of such an impedimetric system, and illustrates an electrochemical well and its associated electronic circuit.

As a result of microbial metabolism weakly charged substances in the initial growth medium are converted to end products of greater charge. These are generally smaller and therefore more mobile and conductive in the bulk fluid. Such changes are the origin of the microbially

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a)

Monitoring instrument

b)

Bulk fluid impedance

Polarisation impedance

Polarisation impedance

Cpol   Rpol

Rsol

Rpol   Cpol

Cathode

Anode
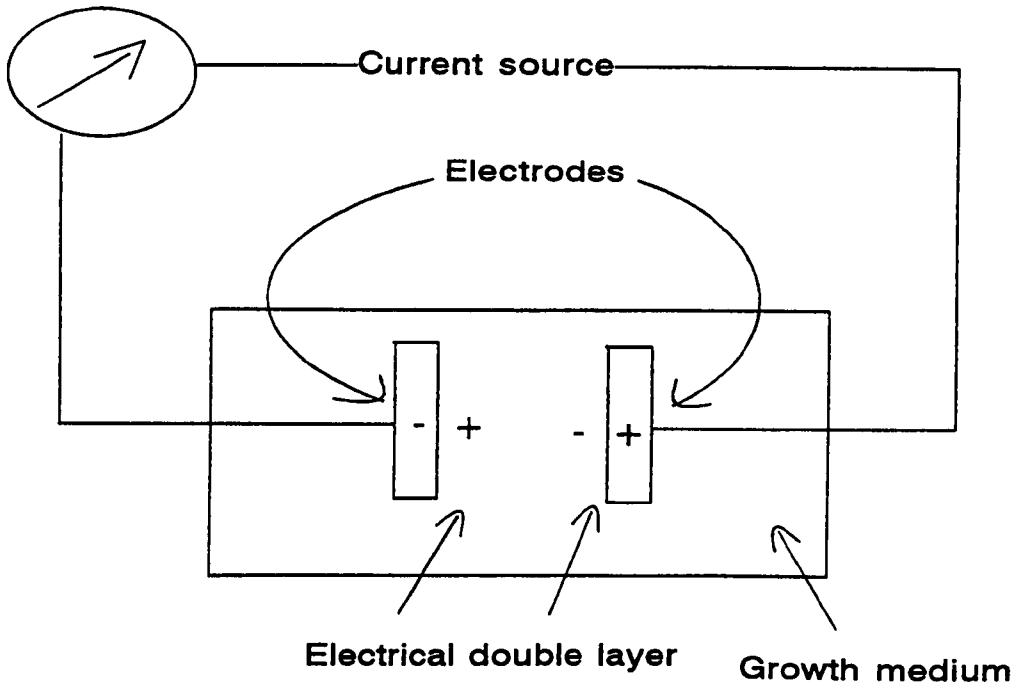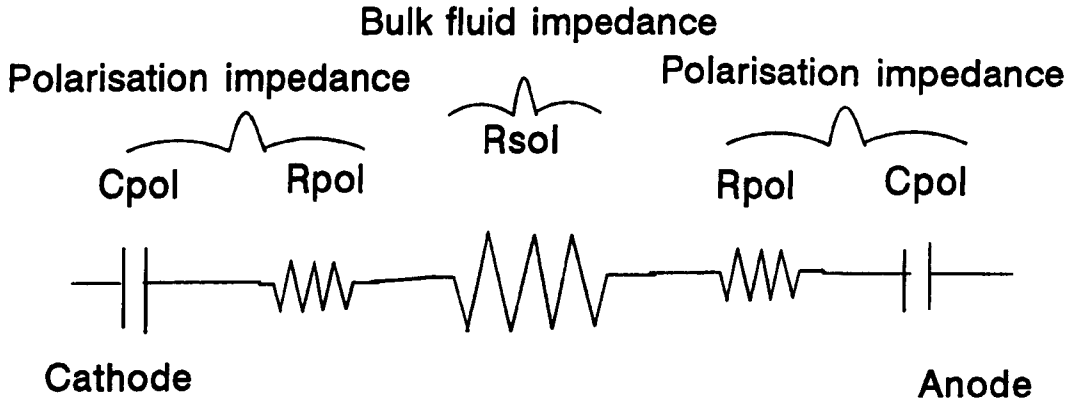
Fig. 1.2 Illustration of a) the position of an electrochemical well within the electric circuit and b) the impedimetric circuit. Key: Cpol= polarisation capacitance, Rpol= polarisation resistance and Rsol= resistance of bulk fluid (modified from Firstenberg-Eden & Zindulis, 1984).

induced conductance signals (Firstenberg-Eden & Zindulis, 1984). Indirect conductimetry, which allows the detection of yeasts and moulds despite the small conductance changes they produce, has also been developed (Owens et al., 1989; Bolton, 1990). This technique assesses samples by absorption of metabolically produced carbon dioxide in an alkaline solution in which conductance changes are then measured. Capacitance changes arise from the accumulation of small metabolic products in close proximity to the electrodes (Firstenberg-Eden & Zindulis (1984). These affect the electrical signals by both reducing the distance within the electrical double layer and reducing the distances between electrodes. Smaller ions can also increase the effective surface area of the electrode by increasing the concentration of ions at the electrode surface. Different metabolites have different dielectric constants which affect capacitance as follows:

$$\text{Capacitance} = E \cdot \frac{A}{4 \, \text{pi} \, d} \qquad \text{(equation 1.1)}$$

> where:
> E = the dielectric constant of the solution between the electrodes and the double layer
> A = area of the dielectric
> d = the thickness of the dielectric

The optimal electrical algorithm for use in the detection of bacteria remains unclear. The changes accompanying bacterial growth and metabolism have been reported to have little effect on capacitance by some

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authors (Richards et al., 1978) whereas others Firstenberg-Eden & Zindulis (1984) have concluded that capacitance can be sensitive to bacterial growth. Impedance has been shown to indicate bacterial growth, the threshold level of detection being approximately 10^6 to 10^7 cells cm^{-3} (Firstenberg-Eden & Zindulis, 1984). Zindulis (1984) stated that while all three electrical parameters are potentially useful for monitoring microbial growth, in practice the optimal algorithm depends on the medium and the target organism.

In summary the production of microbial electrolytic products can affect the conductance, capacitance and impedance components of an electrochemical circuit. This results in changes in the voltage and current applied to the reaction well, which can be monitored by an appropriate monitoring instrument. Hadley and Yajko (1985) equated the relationship between voltage (E) and current (I) for a given frequency (f) when a circuit contains both resistance (R) and capacitance (C) as follows:

$$E = I \ (R^2 + Xc^2 \ )^{\frac{1}{2}} \qquad \text{(equation 1.2)}$$

where Xc, the capacitive reactance is given by:

$$Xc = {}^{1/}(2 \ \text{Pi} \ f \ C)^2. \qquad \text{(equation 1.3)}$$

1.5.2 Instrumentation

Currently in the UK there are three commercially available instruments designed to monitor the growth of microorganisms by electrical measurement. The Bactometer (bioMérieux Limited (Ltd.)), used in this research, measures impedance, conductance and capacitance (Nieuwenhof & Hoolwerf, 1987), the Malthus microbial growth analyser (Malthus instruments) measures conductance only (Hogg et al., 1987), and the RABIT (Rapid Automated Bacterial Impedance Technique, Whitley Scientific Ltd.) measures conductance and admittance (total impedance) (Whitley D., personal communication, 1989). There are two American machines marketed by Honeywell Incorporated, the automated culture growth and detection system and the medical specimen culture bottle system, which both monitor impedance in agitated bacterial cultures. The Japanese Orga 6 automatic microbial analyser (Tektron Instruments Corporation) also monitors impedance (Hadley & Yajko, 1985). In addition two unique non-commercial instruments have been reported. The Goldschmidt and Wheeler System (University of Texas) has been used to monitor non-growing bacteria by electrically measuring washed cells suspended in distilled water, rather than their metabolites (Hadley & Yajko, 1985). The second system, described by Wilkins et al. (1974), detects the evolution of hydrogen from growing cells by an increase in voltage.

Components of the Bactometer are illustrated in Fig. 1.3. The Bactometer Microbial Monitoring unit (M64)
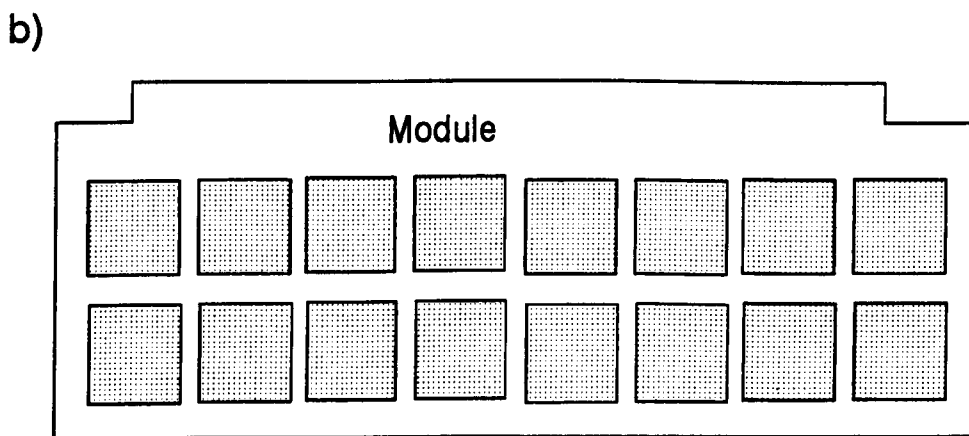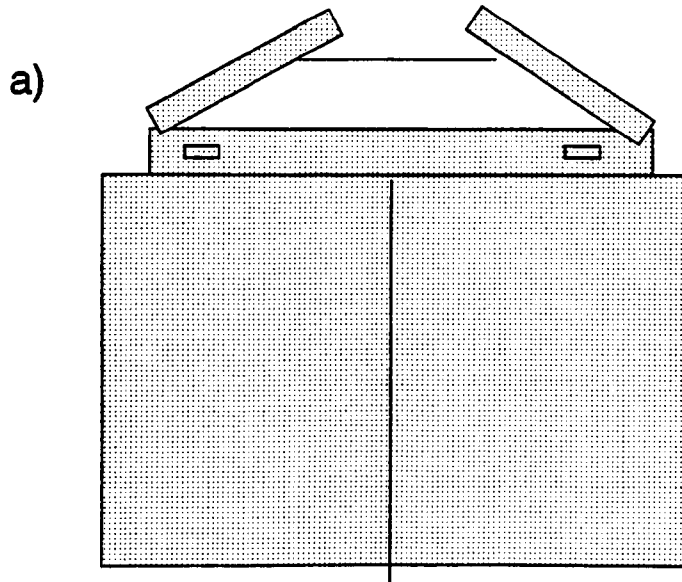
Fig. 1.3 Schematic diagram of a) a Bactometer M64 microbial monitor and b) a module containing 16 reaction wells.

consists of two incubators with independent temperature control linked to an IBM PS/2 desk top computer. Each incubator can accommodate two modules consisting of 16 reaction wells of 2.5 cm^3 capacity each with attached electrodes. The menu driven software is capable of the fully automated monitoring of up to 64 reaction wells and has a variety of options including the display of graphics and data storage. Hard copies of the data generated are obtained from the attached printer.

The commercial instruments monitor electrical changes in one of two ways. In some cases a comparison is made between the inoculated reaction well and an uninoculated reference well, the latter acting as a control for temperature changes, evaporation and degradation of culture medium during incubation (Hadley & Yajko, 1985). The other type of monitoring, used for example in the Bactometer, involves no control wells but instead compares initial impedimetric values with later measurements. A comparative record of percentage change is made and the Bactometer then plots conductance, capacitance or the reciprocal of impedance against time (the latter to provide a positive rather than a negative curve). Fig. 1.4 is an example of such an electrical curve. The stabilisation time is the time required after the sample is placed in the instrument before the baseline is established. This initial variation in the electrical signal is due to fluctuations in temperature, both polarisation capacitance and bulk fluid conductance being sensitive to temperature change before the respective
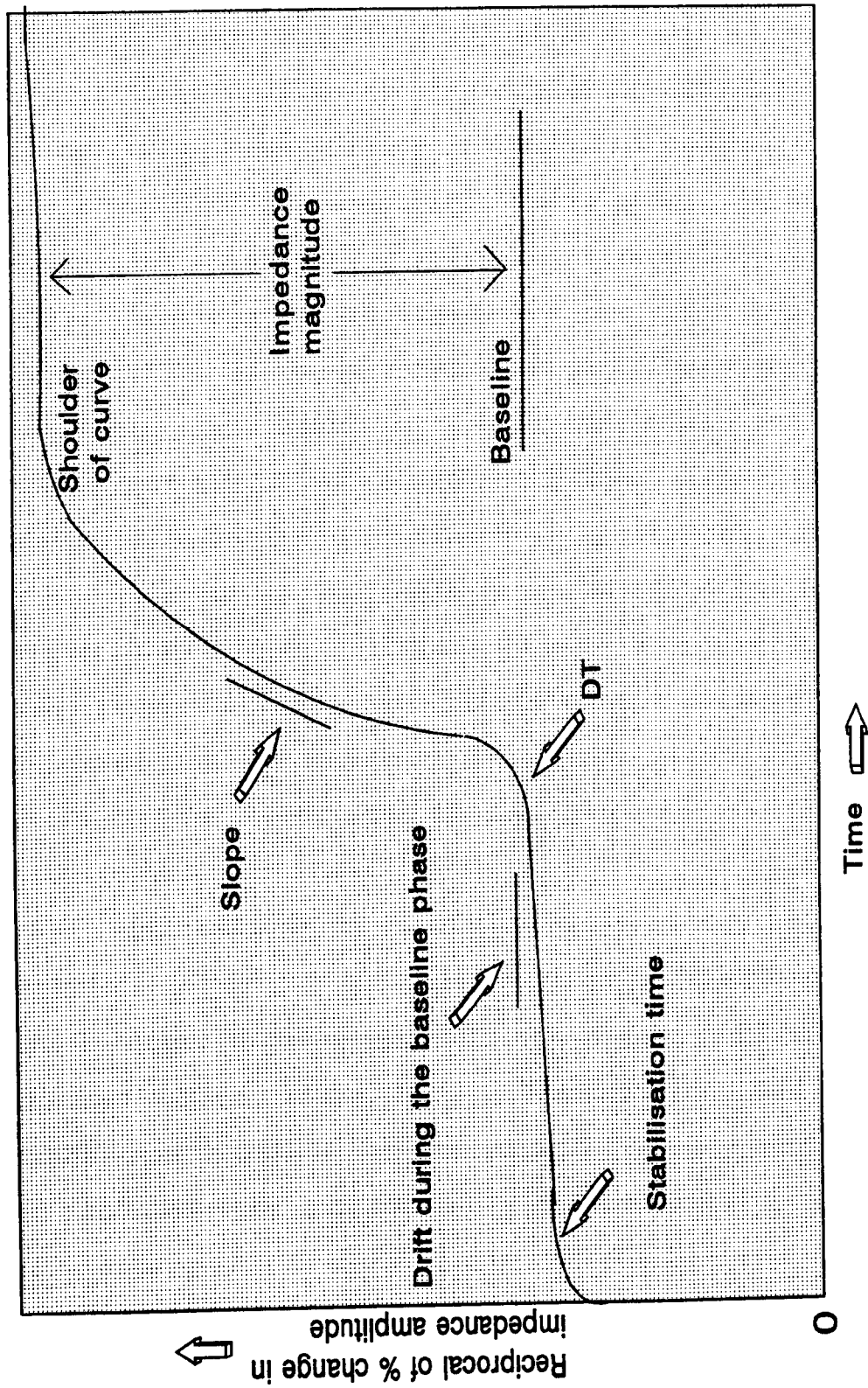
Fig. 1.4 Idealised impedimetric detection curve (modified from Firstenberg-Eden, 1986). DT = detection time.

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equilibria are established (Firstenberg-Eden & Zindulis, 1984). Drift describes the relative change in impedance during the baseline phase. Ideally drift is zero, though in practice the curve usually drifts upwards or downwards. The detection time (DT) is the point of maximum acceleration of the electrical curve and is determined by the Bactometer automated software (Firstenberg-Eden, 1983). The slope is the segment of the curve during which the amplitude of the electrical signal increases with time. This phase starts at the DT and levels out at the shoulder of the curve (Firstenberg-Eden, 1986). Impedance magnitude, as defined by Arnott et al. (1988), corresponds to the increase in amplitude from the DT to the shoulder of the curve. It is important to note an electrical curve is not a growth curve. The relationships between bacterial concentration, ionic concentration and electrical detection are illustrated in Fig. 1.5.

1.5.3 Applications of impedance microbiology

Impedance technology has been applied to fundamental microbiology, medical microbiology and food microbiology (Bossuyt & Waes, 1983). Once the methodology was established impedimetric assays were rapidly adopted by the food industry for routine microbiological testing (Firstenberg-Eden & Eden, 1984) and for quality control analysis (Connolly et al. 1988). Sharpe (1986) reported that it is currently the most highly developed automated technique used in food microbiology because it employs user-friendly computerised systems, the data generated

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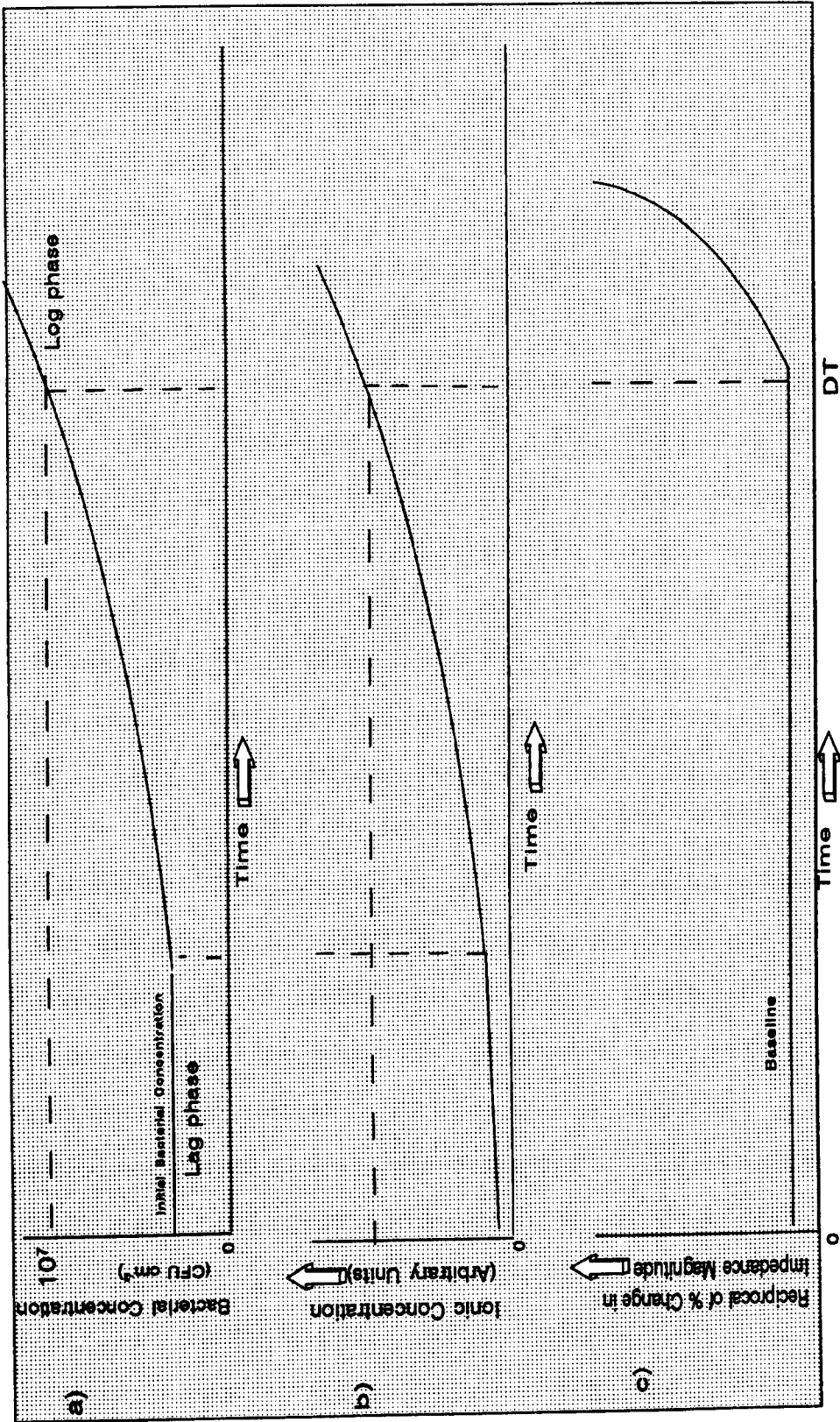
Fig. 1.5 Relationship between bacterial concentration, ionic concentration and impedimetric detection (modified from Firstenberg-Eden, 1986).

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correlate well with conventional plate counts and the most highly contaminated samples are detected first. Furthermore, as Sharpe (1986) also commented, metabolic activity data are more useful than plate counts for shelf life prediction.

Electrical monitoring has been evaluated for both the enumeration and detection of microorganisms. For example, it has been used as an alternative to the plate count method for estimating the total viable count (TVC) of bacteria (Firstenberg-Eden, 1983), for sterility testing of ultra heat treated (UHT) products (Firstenberg-Eden, 1986), for the estimation of bacteria in raw milk (Nieuwenhof & Hoolwerf, 1987), for the estimation of the bacterial quality of fish (van Spreekens & Stekelenberg, 1986) and for the estimation of yeast numbers (Shapton & Cooper, 1984). Bossuyt and Waes (1983) found this technique useful in tracing post pasteurisation contamination. Electrical monitoring has also been used for the detection of specific microorganisms, for example to determine TVC of coliforms in dairy products (Firstenberg-Eden et al., 1984) and for the detection of bacteriophages in cheese starter cultures (Firstenberg-Eden, 1986). More specifically conductance has been used to detect salmonella in confectionery products (Arnott et al., 1988), Cl. botulinum in cured pork slurry (Gibson, 1987) and a wide variety of yeasts important in food spoilage (Connolly et al., 1988). Impedance has been used to estimate numbers of antibiotic resistant Salmonella in cured pork slurry (Gibson, 1988),

yeasts in yogurts and fruit juices (Firstenberg-Eden, 1986), lactic acid bacteria in fruit juices (Firstenberg-Eden, 1986), staphylococci in meats (Firstenberg-Eden, 1986) and in a detection regimen for fungi using the Bactometer (Zindulis, 1984).

Quality control testing for _Salmonella_ using the Malthus conductance method has been given official approval by the Association of Official Analytical Chemists (AOAC). Within the UK this is also approved in BS4285 (British Standard 4285, 1992). As the AOAC is recognised as a worldwide authority on microbiological methods, their approval was a major step forward in the general acceptance of conductimetric microbiological testing by the food industry (Malthus, 1992). Impedimetric assays have also been approved by the American Public Health Association (Hadley & Yajko, 1985). Of the available automated methods, impedimetric microbiology has gained most commercial acceptance (Coppola & Firstenberg-Eden, 1988). Examples of current commercial uses include routine quality control, maintenance within standard limits and in some cases identification of pathogens (bioMérieux, 1991, 1992a; Malthus, 1991; 1992). Anchor foods, Wiltshire, a subsidiary of the New Zealand Dairy Group, uses the Bactometer for quality assurance of a variety of dairy products (bioMérieux, 1992a). The Malthus instrument is used by the New Zealand Kiwi Cooperative Dairy in Hawera for monitoring coliform levels during butter and cheese production (Malthus, 1991) and by the airline Lufthansa to assay for TVC, enterobacteriaceae

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and staphylococci in airline meals (Malthus, 1991). St. Ivel, Bradford carry out all their Salmonella testing using the Malthus AOAC approved conductance method (Malthus, 1992).

The cosmetic industry and the pharmaceutical industry as well as the food industry now employs electrical detection of microorganisms. Companies have found that the automated technique has improved response times to process failures and therefore they achieve faster quality control clearance times. It has also proved to be cost effective (bioMérieux, 1992a). The Body Shop cosmetics chain uses the Bactometer for all of their bacterial and yeast detection tests. They have found the rapid results obtained enable faster decisions, hence improving stock control. Another cosmetic company Creighton reported that use of the Bactometer permitted release of finished stock several days earlier than traditional methods of quality assurance (bioMérieux, 1991). The pharmaceutical company Rhone-Poulenc, which produces industrial enzymes from bacterial or fungal fermentations, has found the Bactometer aids their adherence to the quality assurance standard BS5750 (bioMérieux, 1991).

Impedance microbiology has also been employed in the analysis of antimicrobial compounds. Conductance has been used to determine the cytotoxic effects of bioreductive antimicrobial and radiosensitising agents (Dale & Edwards, 1989), to detect trichothecene mycotoxins and screen for susceptible microorganisms (Adak et al., 1987a; 1987b), as an alternative to the Ames test for the detection of

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mutagens (Forsythe, 1990) and to determine antimicrobial preservative efficacy (DePasquale et al., 1985).

1.6 Aims of current work

The objective of this study was to utilise recent advances in selective impedance microbiology to reduce the presumptive DT of L. monocytogenes in foodstuffs. Although the use of selective media for the electrical detection of L. monocytogenes has been described by others, both previously published procedures, viz. capacitance signal (Phillips & Griffiths, 1989) and indirect conductimetry (Bolton, 1990), have only been applied to pure cultures.

L. monocytogenes was chosen as the target organism for a number of reasons. Firstly this species has been implicated in a number of outbreaks of food poisoning with high mortality rates (McLauchlin et al., 1986; Kvenberg, 1988). Secondly Listeria spp. are good indicators of potential contamination by other food poisoning agents, as they are difficult to eliminate from food production environments because of their ubiquity and their inherent resistance to processing treatments. Furthermore as traditional methods of detecting L. monocytogenes take a minimum of 10 days (McLauchlin & Pini, 1989), there is the need for a more rapid technique to monitor contamination levels in short shelf life food products.

In this work a number of parameters were examined in an attempt to first characterise and then optimise the electrical signals produced by L. monocytogenes. The available Bactometer algorithms (viz. total impedance,

conductance and capacitance) were compared for their effectiveness in monitoring the target species. A number of curve criteria, including DT, magnitude, slope, peak response rate (PRR) and peak response time (PRT) (Firstenberg-Eden, 1983; Connolly et al., 1988), were investigated to determine which electrical signal(s) were most reproducible.

The growth medium used was formulated to allow active metabolism by L. monocytogenes and therefore the rapid impedimetric detection of this species. Similarly, the optimum physical conditions for the growth of L. monocytogenes (viz. the environmental variables of temperature and pH) were evaluated and used to facilitate active metabolism (Kramer & Jones, 1969; Ralovich, 1984; Leighton, 1985).

A variety of growth inhibitors, such as antibiotics, dyes and inorganic chemicals, were next incorporated into the growth medium in an attempt to suppress the metabolism, and hence the electrical signals, of non target species. The selective agents initially chosen for evaluation were those used commonly in Listeria selective media: nalidixic acid (Kramer & Jones, 1969; Leighton, 1985; Doyle & Schoeni, 1986; Buchanan et al., 1987; Farber et al., 1987; van Netten et al., 1988), oxolinic acid (Farber et al., 1988), thallous acetate (Kramer & Jones, 1969; Farber et al., 1988), potassium tellurite (Schoer, 1944; Kramer & Jones, 1969), lithium chloride (Kramer & Jones, 1969; Farber et al., 1987, 1988; Curtis et al., 1989a), phenylethanol (Kramer & Jones, 1969; Farber et

al., 1987; van Netten et al., 1988), acriflavine (Dominguez et al., 1984; Leighton, 1985; Doyle & Schoeni, 1986; Farber et al., 1987, 1988; van Netten et al., 1988) and moxalactam (Buchanan et al., 1987; Bannerman & Bille, 1988; Farber et al., 1988; van Netten et al., 1988).

Growth inhibitors which selectively eliminated the impedimetric responses of organisms other than Listeria spp., without increasing the DT of L. monocytogenes unduly or reducing the quality of its electrical curves, were then chosen for use in the selective impedimetric assay. Selectivity for only pathogenic Listeria spp. was the eventual goal.

Attempts were also made to determine the biochemical basis of the detection of L. monocytogenes. Quantification of a number of ionic products of metabolism (including organic acids and ammonia) was undertaken using both commercially available test kits and gas chromatography.

CHAPTER 2

MATERIALS AND METHODS

2.1 Test Strains

The organisms used in this work are listed in Table 2.1.
The bacterial species were maintained on nutrient agar
No. 1 (Oxoid) slopes at 4 °C and routinely subcultured at
3 monthly intervals. The identity of cultures was
confirmed at 6 monthly intervals using Api test kits
(bioMérieux Ltd., Basingstoke, UK). The ten Listeria food
isolates were identified by Heckfield Laboratories,
Basingstoke, UK, as follows: Isolates I1 and I2 as either
non haemolytic L. monocytogenes or L. innocua; isolates
I3 to I10 as haemolytic L. monocytogenes. Fungal species
were maintained on malt extract agar (Oxoid) slopes at
4 °C and routinely subcultured at 3 monthly intervals.

2.2 Microbiological Media

Glucose enriched nutrient broth (GNB) was used both as a
general growth medium and as the basal impedimetric
medium. It contained per litre: bacteriological peptone
(Oxoid), 10 g; lab-lemco powder (Oxoid), 10 g; sodium
chloride (BDH Biochemicals), 5 g; glucose (BDH
Biochemicals), 2 g. GNB was sterilised by autoclaving at
121 °C for 15 minutes.

Where appropriate GNB was supplemented with one or
more antimicrobial agents. Thallous acetate (BDH
Biochemicals), phenylethanol (Sigma), potassium tellurite

Species	Source and strain number	Gram stain	L. monocytogenes serotype
L. monocytogenes	NCTC 7973	+	1a
L. monocytogenes	NCTC 10357	+	1a
L. monocytogenes	NCTC 5348	+	2
L. monocytogenes	NCTC 10527	+	4b
L. monocytogenes	NCTC 11994	+	4b
L. innocua	NCTC 11288	+	
L. innocua	NCTC 10889	+	
L. welshimeri	NCTC 11957	+	
L. grayi	NCTC 10815	+	
L. murrayi	NCTC 10812	+	
L. ivanovii	NCTC 11846	+	
L. seeligeri	NCTC 11856	+	
Listeria spp.	10 Food isolates, (I1 to I10) obtained from bioMérieux Ltd Basingstoke, UK	+	Unknown
Jonesia denitrificans	NCTC 10816	+	
Lactococcus lactis	NCIB 6681	+	
Lact. lactis subsp cremoris	NCIB 8662	+	
Enterococcus faecalis	NCIB 775	+	
Streptococcus agalactiae	NCIB 8778	+	
Strep. salivarius	NCIB 8883	+	
Leuconostoc mesenteroides subsp cremoris	NCIB 12008	+	
Lactobacillus casei subsp rhamnosus	NCIB 6375	+	
Micrococcus roseus	NCIB 8175	+	
Bacillus cereus	NCIB 8122	+	
B. circulans	NCIB 7578	+	
B. subtilis	NCIB 8703	+	
Pseudomonas fluorescens	NCIB 3756	−	
Ps. putida	NCIB 10936	−	
Ps. aeruginosa	NCIB 950	−	
Ps. aeruginosa	NCIB 10848	−	
Escherichia coli	NCIB 86	−	
Penicillium roquefortii	NCTC 588	N/A	
P. claviforme	ATCC 10426	N/A	

Key

N/A = Not applicable
NCIB = National Collection of Industrial Bacteria
NCTC = National Collection of Type Cultures
ATCC = American Type Culture Collection
subsp = subspecies

Table 2.1 Test strains of microorganisms used

(BDH Biochemicals), oxolinic acid (Sigma), amphotericin B (Sigma) and ketoconazole (Sigma) were added to the medium before sterilisation. Depending upon the individual experiment, nalidixic acid (Sigma) was either added to the medium before sterilisation or filter sterilised prior to addition to sterilised medium. The latter procedure was also used for moxalactam diammonium salt (Sigma), moxalactam sodium salt (Sigma), acriflavine hydrochloride (Sigma), proflavine hydrochloride (Sigma) and lithium chloride (Hopkin & Williams). These agents were dissolved in distilled water and filter sterilised by the use of 0.22 μm filters (Sartorius) and positive pressure. As certain selective agents lost their activity with time after addition to GNB (I. Hancock, unpublished work), the medium was always supplemented immediately prior to use.

Aesculin nutrient broth (ANB) was prepared by substituting aesculin (Sigma), 1 g dm^{-3}, and ammonium-ferric citrate (Griffin & George), 1 g dm^{-3}, for the glucose component of GNB. These concentrations were chosen as they had been reported to be effective by Dominguez et al. (1984).

L-rhamnose nutrient broth (RNB) was prepared by substituting L-rhamnose (Sigma), 2 g dm^{-3}, for the glucose component of GNB. Although Rocourt et al. (1983) recommended 10 g dm^{-3} L-rhamnose, 2 g dm^{-3} was used here to avoid increasing the total concentration of carbohydrate in the medium.

Media were normally adjusted to pH 7.2, the reported

optimum for <u>L. monocytogenes</u> (Kramer & Jones, 1969), by
the addition of 0.1 M sodium hydroxide (BDH Biochemicals)
or 0.1 M hydrochloric acid (BDH Biochemicals). pH values
were measured by a pH meter (Cranwell model 990). Where
adjustments were made to previously sterilised media, the
pH probe itself was sterilised by immersion in absolute
ethanol for 1 minute followed by immersion in sterile
distilled water for 1 minute.

2.3 Routine Growth of Microorganisms

A loopful of material from the appropriate stock slope
was aseptically added into a universal bottle containing
10 cm^3 of GNB. The culture was then incubated at 30 °C
for 24 h. Such 24 h cultures were used as inoculum for
all spectrophotometric and impedimetric assays. In the
latter case a 10^{-4} dilution of the 24 h culture in 1/4
strength Ringers solution (BDH Biochemicals) was normally
made first. However, if only sparse growth was noticed
after 24 h incubation a 10^{-2} dilution was used instead.
For detection of very low levels of <u>L. monocytogenes</u>, a
10^{-7} dilution of the 24 h culture in 1/4 strength Ringers
solution was used to inoculate the enrichment medium.

Normally mixed cultures were prepared by the
addition into a sterile vessel of 1.0 cm^3 of a 24 h GNB
culture of each test microorganism diluted to 10^{-3} in 1/4
strength Ringers solution. The vessel was then made up to
10 cm^3 with 1/4 strength Ringers solution to produce a
10^{-4} dilution of each species within the mixed culture.

2.4 Cell Enumeration

Bacterial concentrations were determined on nutrient agar No. 1 (Oxoid) using the pour and/or spread plate techniques. Fungal concentrations were similarly determined using malt extract agar (Oxoid). 0.1 cm^3 and/or 1 cm^3 aliquots of a range of dilutions were used to inoculate plates. Gilson micropipettes (Anachem, Luton, Bedfordshire) were used throughout this work. The plates were incubated at 30 °C for 24 to 48 h and then those containing between 30 and 300 colonies were counted. The TVC was determined by multiplying the number of CFU by the reciprocal of the dilution factor and the reciprocal of the aliquot volume used as inoculum. The standard deviation (SD) of at least quadruplicate plates were then determined (see 2.14).

Where appropriate impedimetric data were confirmed by spread plating on <u>Listeria</u> selective agar (Oxoid Oxford formulation base with <u>Listeria</u> selective supplement SR140E).

2.5 Spectrophotometric Assay of Microbial Growth

Initial screening of the antimicrobial agents was performed by spectrophotometric assay. In each test, a tube containing 10 cm^3 of GNB supplemented, where required, with the appropriate agent(s) was inoculated with a 0.1 cm^3 aliquot of a 24 h GNB culture of the test microorganism (see 2.3) and incubated at 30 °C for 24 h. The absorbance of the culture was then determined at the

wavelength of maximum absorbance using an ultra violet /
visible spectrophotometer (Cecil instruments). The higher
the absorbance value, the greater the extent of growth
during the incubation period and hence the greater the
resistance of the test species to the antimicrobial
agent(s).

2.6   Impedimetric Assay of Microbial Growth

A Bactometer M64 Microbial Monitoring System (bioMérieux
Ltd., Basingstoke, UK) was used for the impedimetric
assays (Fig 1.3). In this instrument four modules, each
with 16 reaction wells, can be run simultaneously giving
a total of 64 wells. For routine screening of selective
agents, each well containing 1 cm^3 GNB supplemented,
where required, with the appropriate antimicrobial
agent(s) was inoculated with a 0.1 cm^3 aliquot of a 24 h
GNB culture of the test microorganism diluted in 1/4
Ringers solution (see 2.3). The electrical responses were
monitored for 36 h at 30 °C. Normally impedimetric
responses (standard impedance algorithm 1) were
determined. However, on occasion, conductance (standard
conductance algorithm 2) or capacitance (standard
capacitance algorithm 3) responses were also monitored.

2.6.1 Curve interpretation

DT and curve magnitude were the criteria established for
the detection of the target species. The Bactometer
registers the DT at the point of maximum rate of change
in the electrical properties of the medium. Curve

magnitude was measured by subtracting the percentage
change in amplitude of the electrical signal at the DT
from the percentage change in amplitude at the shoulder
(Fig. 1.4) and then multiplying by the baseline value. If
a shoulder was not apparent, then the maximum magnitude
was determined instead. Magnitude was calculated in
arbitrary units of impedance (BUIC), conductance (BUCO)
or capacitance (BUCA) as in the report of
Firstenberg-Eden (1983). For example, to calculate
magnitude in arbitrary units of impedance:

Difference in percentage change in amplitude	=	percentage change in amplitude at shoulder	−	percentage change in amplitude at DT	(equation 2.1)

$$= 45\ \% - 8\ \%$$

$$= 37\ \%$$

Baseline value $\quad = 2476$

Therefore,
impedance magnitude $\quad = \dfrac{37}{100}\ x\ \ 2476$

$$= \underline{916\ BUIC}$$

The shorter the DT the greater the extent of growth
during the incubation period and hence the greater the
resistance of the test species to the antimicrobial
agent(s) provided the initial inoculum concentration is
kept constant.

2.6.2 Correlation between impedimetric data and plate count data

The impedimetric responses and the TVC (see 2.4) of

serial dilutions (10^{-1} to 10^{-8} in 1/4 strength Ringers solution) of L. monocytogenes were simultaneously examined. A graph of DT against TVC was then plotted and subjected to correlation analysis (see 2.14). Where correlation was found to be significant and repeatable, the data from several such experiments were combined to produce a calibration curve from which a TVC could be predicted from an experimentally determined DT value. Separate calibration curves were produced for each strain of L. monocytogenes and each medium / selective agent combination.

2.7 Detection of L. monocytogenes in Food Products

A direct impedimetric assay for the detection of L. monocytogenes in foods was developed. A variety of cheeses (freshly purchased and stored at 4 °C during transport) were artificially infected (spiked) with L. monocytogenes as follows: Cheese suspensions (10 % (w/v) in 1/4 strength Ringers solution) were prepared and dispersed in a Colworth stomacher for one minute. For routine screening, 10 cm^3 suspensions were spiked with 0.1 cm^3 of an appropriate dilution of a 24 h GNB culture of L. monocytogenes in 1/4 strength Ringers solution (see 2.3). The spiked food matrix was then vortex mixed (Jencons Whirlimixer). A 0.1 cm^3 aliquot was inoculated into a Bactometer well containing 1.0 cm^3 of GNB supplemented, where appropriate, with selective agent(s). Using routine conditions (see 2.6 & 2.6.1) the impedimetric responses were monitored for 36 h and the DT

and impedance magnitudes determined.

All the cheeses used in this work were screened for the presence of naturally occuring <u>L. monocytogenes</u> using a traditional <u>Listeria</u> selective agar method (see 2.4). Any positive isolates from non spiked food samples were identified using API Zym identification kits (bioMérieux Ltd., Basingstoke, UK).

2.8 Selective Enrichment for <u>L. monocytogenes</u>

The aim of enrichment was to allow the target organism to multiply to a level where a 0.1 cm^3 sample contained a sufficiently high cell concentration to be detectable by the impedimetric assay. A number of regimens were evaluated for zero tolerance assurance, viz. the detection of one cell of <u>L. monocytogenes</u> in 25 g of a foodstuff. The variables investigated were the concentration of cheese suspension, the concentration of selective agents in the enrichment medium, the pH of the enrichment medium, the enrichment time and the volume transferred from the enrichment vessel into the Bactometer well.

As a result of these trial experiments the following procedure was developed: 5 % (w/v) cheese suspensions were prepared by adding 25 g cheese to 475 cm^3 of GNB and homogenising for 1.0 minute in a Colworth stomacher. The suspensions were aseptically adjusted to pH 7.2 (see 2.2), sterilisation and cleaning of fatty materials from the pH probe being achieved by its immersion and rinsing

with absolute ethanol for one minute followed by immersion in sterile distilled water for one minute. The suspensions were then aseptically supplemented with the selective agents, 30 mg dm^{-3} proflavine hydrochloride and 30 mg dm^{-3} moxalactam. Parallel enrichment vessels were set up at zero time, one enrichment vessel remained unspiked, while a second was spiked with a 24 h GNB culture of an appropriate strain of L. monocytogenes diluted to 10^{-7} in 1/4 strength Ringers solution (see 2.3). At the same time the 24 h GNB culture was enumerated by routine methods (see 2.4). The enrichment vessels were then incubated at 30 °C for 24 h. At the end of this incubation period appropriately diluted 0.1 cm^3 samples from each enrichment vessel were transferred to 2.2 cm^3 of freshly prepared doubly supplemented GNB in Bactometer wells. Using routine conditions (see 2.6 & 2.6.1), their impedimetric responses were then monitored for 36 h and the DT and impedance magnitudes determined.

2.9 Aesculin Hydrolysis Test for L. monocytogenes

A second detection test for L. monocytogenes was evaluated for running concurrently with the impedimetric assay. Certain species, including the target organism, can hydrolyse the glycoside aesculin if iron salts are included in the growth medium. Hydrolysis can then be observed by monitoring the formation of a black pigment.

1.0 cm^3 of ANB (see 2.2), supplemented when required with selective agents, was inoculated with 0.1 cm^3 of a 24 h GNB culture of an appropriate strain of

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<u>L. monocytogenes</u> diluted in 1/4 strength Ringers solution
(see 2.3). Using routine conditions (see 2.6 & 2.6.1),
the impedimetric responses were monitored for 36 h and at
the same time any colour change in the medium was noted.

2.10 Preparation of Cell Free Extracts of Culture Medium for Biochemical Analysis

10 cm^3 of GNB was inoculated with 0.1 cm^3 of a 24 h GNB
culture of an appropriate test strain diluted to 10^{-3} in
1/4 strength Ringers solution. The diluted cells were
incubated at 30 °C in a culture vessel. After 24 h
incubation the cell concentration in the culture vessel
was measured (see 2.4) and then a cell free extract was
prepared by filter sterilising the remaining culture
medium using a 0.22 μm filter (Sartorius).

2.11 Spectrophotometric Method for the Determination of L-Lactic Acid

The concentration of L-lactic acid was determined using a
L-lactic acid test kit (Boerhinger-Mannheim). In this
procedure L-lactic acid is oxidised by nicotinamide
adenine dinucleotide (NAD) in the presence of L-lactic
acid dehydrogenase (Noll, 1974; Sigma diagnostic kit
schedule):

(L-lactic acid dehydrogenase)

L-lactic acid + NAD^+ ←------→   pyruvate + NADH + H^+ (equation 2.2)
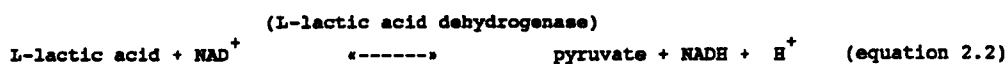
As the equilibrium of the reaction is predominantly to the side of L-Lactic acid, a second reaction, catalysed by the enzyme glutamate-pyruvate transaminase, is used to trap the pyruvate and shift the equilibrium in favour of pyruvate and reduced nicotinamide adenine dinucleotide (NADH).

(equation 2.3)

(glutamate-pyruvate transaminase)

pyruvate + L-glutamate   ←------→   L-alanine + 2-oxoglutarate
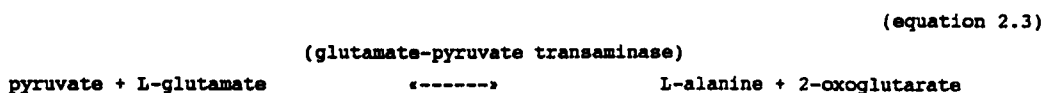
The concentration of NADH is monitored by its absorbance at 340 nm. This can then be related to the concentration of L-lactic acid, as the amount of NADH formed is stoichiometric with the amount of L-lactic acid oxidised (equation 2.2).

The lactic acid test kit contained 4 reagents: solution 1, glycylglycine buffer, pH 10; L-glutamic acid, 440 mg; stabilisers; solution 2, 210 mg NAD lyophilisate, dissolved in 6 cm^3 of double distilled water; solution 3, 0.7 cm^3 glutamate-pyruvate transaminase suspension, 1100 U; solution 4, 0.7 cm^3 L-lactate dehydrogenase solution, 3800 U.

Cell free extracts (see 2.10) were heated to 80°C for 15 minutes to stop enzymic reactions. The concentration of L-lactic acid in these extracts, freshly prepared GNB and a standard solution of L-lactic acid (0.195 g dm^{-3}) was then measured as follows. For each

test performed, the following were added in order into a glass cuvette: solution 1, 1.0 cm^3; solution 2, 0.2 cm^3; double distilled water, 0.9 cm^3; solution 3, 0.02 cm^3; sample, 0.1 cm^3. The contents of the cuvette were mixed and, after approximately 5 minutes at room temperature, their absorbance at 340 nm was measured against a distilled water blank using a ultra violet/visible spectrophotometer (Philips, model PU 8750) (absorbance A1). A reagent blank was similarly set up except the 0.1 cm^3 sample was replaced by 0.1 cm^3 of double distilled water. The absorbance of the contents of this cuvette were also measured after 5 minutes at room temperature (absorbance B1). The reaction was then started in both cuvettes by the addition of 0.02 cm^3 of solution 4. The contents of each cuvette were mixed and left at room temperature for approximately 20 minutes for the reaction to be completed. After this time the absorbance of both the sample (absorbance A2) and the reagent blank (absorbance B2) was measured at 340 nm against a distilled water blank. The absorbance difference for both the sample (A2 - A1) and the reagent blank (B2 - B1) was determined, and then the latter value was subtracted from the former to obtain the change in absorbance (Δ A)

viz: Δ A   = (A2 - A1) - (B2 - B1)   (equation 2.4)

All samples were appropriately diluted in double distilled water to ensure that the absorbance difference

was at least 0.1 units in each case and that no
absorbance reading was above the maximum measurable by
the spectrophotometer.

The concentration of L-lactic acid could then be
determined using the following formula:

$$c = \frac{V \times mol.\ wt.}{E \times d \times v \times 1000} \times \Delta A \quad \text{(equation 2.5)}$$

Where:
c = concentration (g dm^{-3})
V = final volume (2.24 cm^{-3})
v = sample volume (0.1 cm^{-3})
d = light path (1.0 cm)
E = absorption coefficient of NADH at 340 nm (6.3)
mol. wt. = molecular weight of L-lactic acid
(90.1 g mol^{-1})

Therefore:

$$c = \frac{2.24 \times 90.1}{6.3 \times 1.0 \times 0.1 \times 1000} \times \Delta A = \frac{201.8}{630} \times \Delta A = 0.320 \times \Delta A \ \text{g dm}^{-3}$$

For example to calculate the concentration of L-lactic
acid in a solution diluted 1/10 in double distilled
water, where:

A1 = 0.432
A2 = 0.647
B1 = 0.408
B2 = 0.499

$$\Delta A = (0.647 - 0.432) - (0.499 - 0.408)$$
$$= 0.215 - 0.091$$
$$= 0.124$$

Therefore c = 0.320 x ΔA x dilution factor
= 0.320 x 0.124 x 10
= <u>0.397 g dm^{-3}</u>

2.12   Spectrophotometric Method for the Determination of Ammonia

The concentration of ammonia was determined using a modified urea nitrogen test kit (Sigma). In this procedure the Berthelot reaction is used. In the presence of a nitroprusside catalyst, ammonia reacts with an alkaline solution of sodium hypochlorite and phenol to form indophenol which has an intense blue colour (Kaplan, 1965; Martinek, 1969; Sigma diagnostic kit schedule).

$$NH_3 + phenol\text{-}hypochlorite \xrightarrow{\quad nitroprusside \quad} indophenol \quad (equation\ 2.6)$$
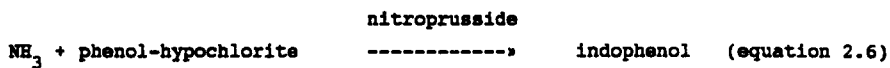
The absorbance of indophenol is measured spectrophotometrically at 570 nm and related to the concentration of ammonia using a predetermined calibration curve.

To tubes containing 0.1 cm^3 aliquots of a) undiluted cell free extracts (see 2.10), b) cell free extracts diluted to 10^{-1} in distilled water, c) freshly prepared undiluted GNB or d) GNB diluted to 10^{-1} in distilled water, were added the following reagents: 1.0 cm^3 of phenol nitroprusside solution, 1.0 cm^3 of alkaline hypochlorite solution and 5.0 cm^3 of distilled water. The contents of the tubes were mixed and left at room temperature for 20 to 30 minutes for the colour to develop. The absorbance of each sample was then measured at 570 nm against a distilled water blank using a spectrophotometer (Cecil, model CE 2343D). The absorbance

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of a range of standard concentrations (1.8 to 18 mg dm^{-3}) of ammonium chloride (BDH Biochemicals) in distilled water was determined by the same procedure and used to produce a calibration curve. The concentration of ammonia in the samples was then determined by reference to this graph.

2.13 Chromatographic Method for the Determination of Organic Acids

For chromatographic analysis the procedure of Carlsson (1973) was used. 5 g of cation exchange resin (Dowex, 50W - X8, hydrogen form, particle size 0.39 to 1.0 mm, 16 to 40 mesh, standard grade) (BDH Biochemicals), was hydrated overnight in 200 cm^3 of double distilled water. Mini cation exchange resin columns were then prepared by packing 1.0 cm^3 volumes of the hydrated resin onto glass wool in pasteur pipettes. 0.5 cm^3 aliquots of cell free extracts (see 2.10), freshly prepared GNB and GNB spiked with known concentrations of acids were allowed to drain through the resin. The columns were then washed twice with 0.5 cm^3 of distilled water. All effluents were collected and directly analysed in the gas chromatograph.

The following standard solutions of acids were prepared: 5.2 g dm^{-3} acetic acid (BDH Biochemicals); 4.9 g dm^{-3} proprionic acid (Sigma); 4.8 g dm^{-3} butyric acid (BDH Biochemicals); 4.7 g dm^{-3} pentanoic acid (Sigma); 4.6 g dm^{-3} hexanoic acid (Sigma); 4.6 g dm^{-3} heptanoic acid (Sigma); 9.01 g dm^{-3} lactic acid (BDH Biochemicals).

Routinely, 1 μl of the standards and samples were injected into the gas chromatograph. The standards were further diluted to determine the lowest concentration of the acids detectable by the chromatographic method.

The gas chromatographic conditions were as follows:
Gas chromatograph (Carlo Ebra Strumentazione, model ICU 600):
Temperature
Injector (275 °C)
Flame ionisation detector (300 °C)
Column Chromosorb OV101 (isothermal, 250 °C)

Gas flowrates
Carrier, nitrogen (180 kPa)
Hydrogen (100 kPa)
Air (50 kPa)
Attenuation 09

Chart recorder (Philips Model PM 8251A)
Speed (5 minutes cm^{-1})
Range (10 mV x 1)

2.14   Statistical Analysis

t-Tests were carried out using Biostats software (written by Dr. B. Giles, University of North London). Calculated values of t were compared to tables of t (Murdoch & Barnes, 1986a) for the determination of statistical significance. SD and correlation coefficients (r) were determined by Harvard Graphics Software, version 3.0

(Software Publishing Corporation, Bitstream Incorporated, Copyright 1991). Calculated values of r were compared to tables of r (Murdoch & Barnes, 1986b) for the determination of statistical significance.

2.15   Safety Considerations

Safety was a major consideration in this research. The COSHH guidelines (1988) were strictly adhered to. Aseptic microbiological techniques were used throughout. All microbiological waste was sterilised by autoclaving at 121 °C for 15 minutes in a bench top autoclave (Denley). Other contaminated material was disinfected by application of Hycolin (William Pearson) or 70 % industrial methylated spirits (Charles Tennant & Company). In addition, care was taken to avoid contact, by inhalation or skin contact, with the potentially hazardous antimicrobial agents used. All liquid and solid waste material containing these agents was collected for incineration with afterburn by a specialist disposal company, as recommended by Sigma Pharmaceuticals.

CHAPTER 3

EVALUATION OF GLUCOSE ENRICHED NUTRIENT BROTH
AS AN IMPEDIMETRIC MEDIUM FOR THE DETECTION AND
ENUMERATION OF L. monocytogenes

3.1 Introduction

The basal contents of an impedimetric medium must fulfil

two criteria: (a) they must allow rapid metabolism and

hence rapid detection of the target organism,

L. monocytogenes in this case, and (b) they must enable

the target organism to produce good quality electrical

curves. To achieve these objectives both the chemical

composition of the medium and the physical conditions of

its incubation are manipulated to allow an optimal

electrical signal from the target organism and reduce or

eliminate any electrical signals from non-target

organisms.

There are a number of differences between a selective

impedimetric assay and a traditional microbial assay for a

target organism. This was recognised by Adak et al.

(1987b) who observed microbiological growth in reaction

wells which gave no impedimetric response, and Gibson

(1987) who reported that media used for the isolation of

target organisms by traditional methodology are not

necessarily useful for impedimetric type assays. While

traditional assays are based on growth under selective

conditions, impedimetric microbiology relies on the morphological characteristics of the electrical curves produced. A number of parameters influence these characteristics including the algorithm used to monitor the electrical change, the medium constituents, the metabolism of organisms in the sample being assayed, any buffers present and proton flux (Owens, 1985).

There are two impedimetric techniques which can be used to detect a specific organism. These will be referred to as qualitative and quantitative type assays. The qualitative type assay relies on the production of an electrical curve of characteristic morphology by the target organism, the curve shape differentiating positive from negative detection (see Fig. 3.1, modified from Phillips and Griffiths, 1989). To establish values that could differentiate organisms Arnott et al. (1988), Pugh et al. (1988) and Pettipher & Watts (1989) used as criteria the magnitude of the curve from DT to the shoulder and the overall rate of acceleration of the slope. Positive detection of Salmonella was indicated by a magnitude in excess of 250 BUCO and a rate of 25 BUCO h^{-1}. Connolly et al. (1988), on the other hand, used as curve criteria DT (defined in this case as the time at which microbial metabolism leads to significant change in the electrical signal), PRR (defined as the maximum rate of change of the electrical signal) and PRT (defined as the time at which PRR occurs). PRR is therefore the same parameter as DT as defined by Firstenberg-Eden (1983) and as used in this work. The second type of impedimetric
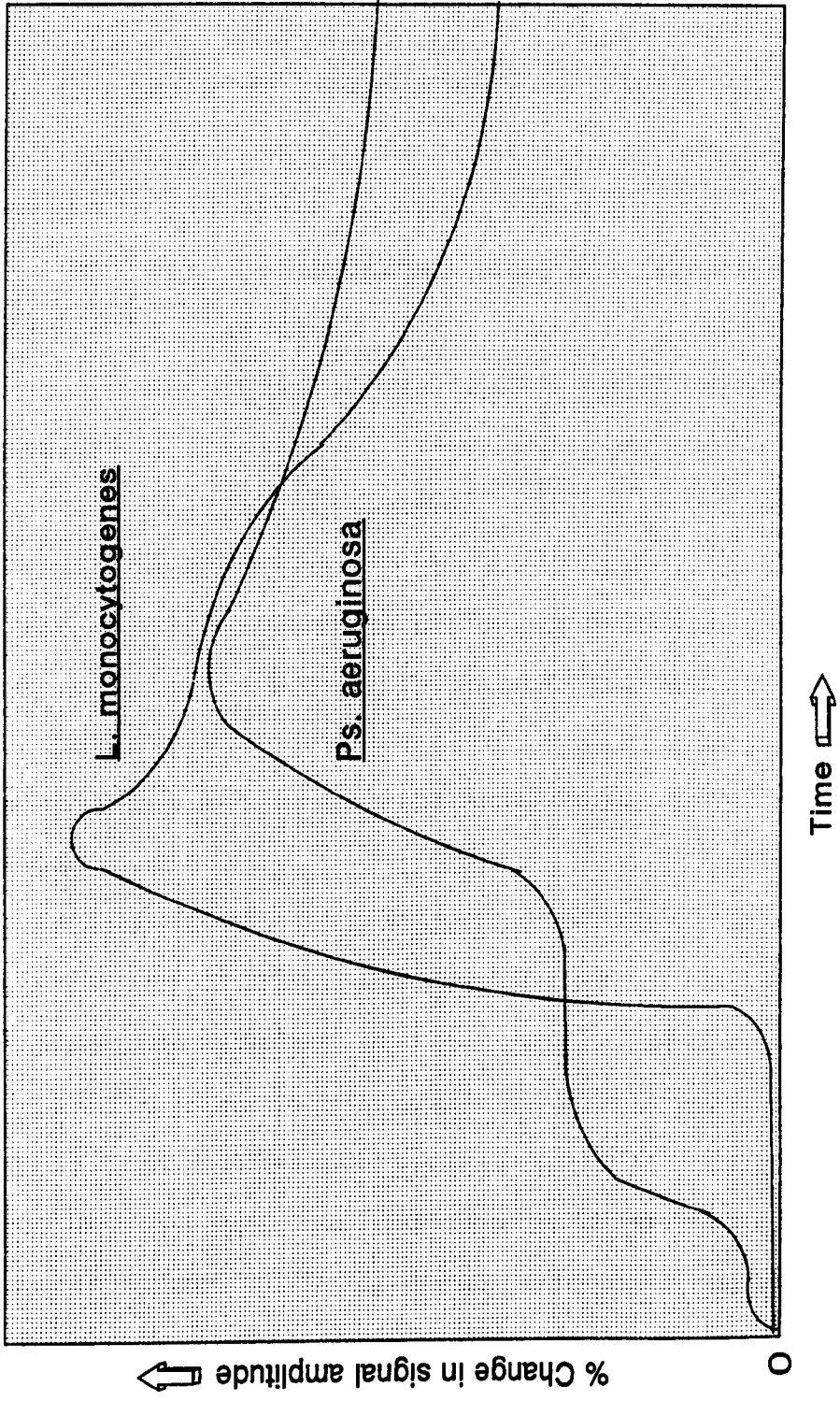
Fig. 3.1 Electrical curves illustrating a qualitative type assay for the detection of L. monocytogenes and, for comparison, Ps. aeruginosa, the morphology of a curve indicating species (modified from Phillips & Griffiths, 1989).

assay is quantitative and involves detection of the target

organism by the presence or absence of an electrical

signal and hence the major criterion for detection is the

production of a DT (Hancock et al., 1993). To accomplish

this the metabolism of all non-target organisms must be

selectively inhibited to such a degree that within the

time limit set no non-target organism is capable of

producing an electrical curve (Fig. 3.2). However, to

assist the detection of the target organism the slow

metabolism of non-target species may be useful for the

production of charged elements at levels below the

threshold of detection of the instrument. As the name

implies a major advantage of the quantitative type assay

is its ability to enumerate as well as detect a target

organism using the inverse relationship between cell

concentration and DT (Firstenberg-Eden, 1983; Nieuwenhof &

Hoolwerf, 1987).

As the success of these electrical techniques relies

on the curves obtained the importance of their quality

cannot be overstressed (Arnott et al., 1988). Curves of

poor quality make it difficult to distinguish or detect

microbial metabolism and to interpret DT. They therefore

cannot be used with any confidence in enumeration. The

amplitude of the curve is important as it is far easier to

distinguish microbial growth when large electrical changes

are produced (Hogg et al., 1987; Connolly et al., 1988;

Silley, 1991). In addition to low amplitude there are
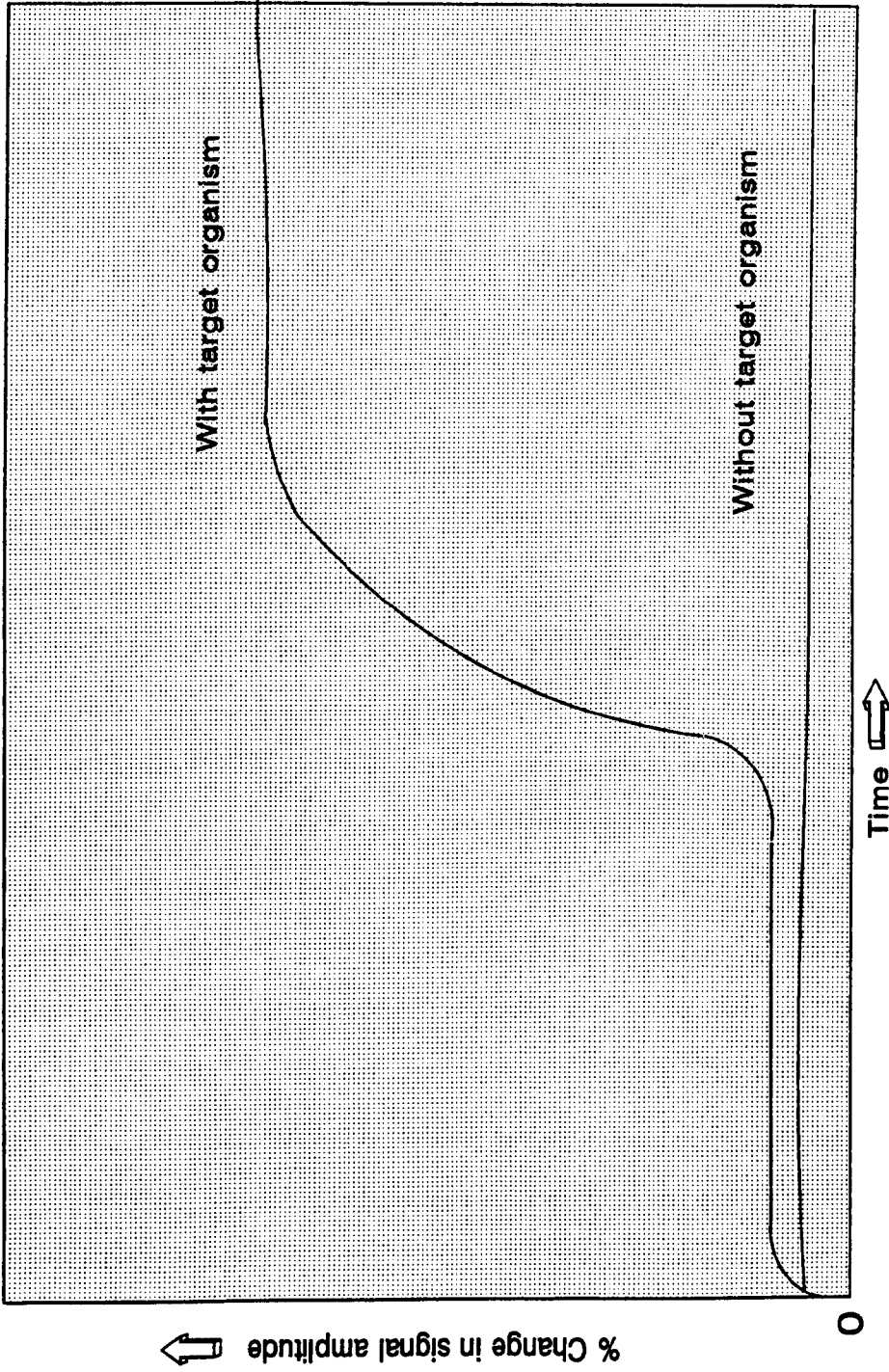
Fig. 3.2 Electrical curves illustrating a quantitative type assay for the detection of a target organism (such as L. monocytogenes), the production of a curve as opposed to a flat baseline indicating the presence of the target species.

a number of other types of poor quality electrical curve
which can be avoided by careful formulation of the
impedimetric medium. These include round acceleration, two
accelerations, weak acceleration and noisy curves.
Examples of each type are illustrated in Fig. 3.3
(modified from Firstenberg-Eden, 1986). Curves
characterised by minimal drift and maximal slope are
optimal as they have more repeatable DT's
(Firstenberg-Eden, 1986). It should be noted, however,
that as most formulations fail to produce satisfactory
curves, the production of a good impedimetric medium has
been described as a difficult task (Arnott et al., 1988).

In 1985 Owens commented that as the theory of
microbially induced electrical changes is poorly
developed, it is not possible to tell if an organism in a
given medium will increase or decrease the conductance.
More recently it has been found that bacteria generally
increase conductance whereas fungi decrease this
electrical signal (Connolly et al., 1988), possibly by the
uptake of ions (Firstenberg-Eden & Zindulis, 1984).
Conductance is regarded as being a less reliable means of
detecting fungal growth than capacitance as only the
latter signal undergoes significant change
(Firstenberg-Eden & Zindulis, 1984). Both capacitance and
conductance (the two components of impedance) are
indicative of bacterial growth. In more conductive media,
however, relative changes in conductance can be small and
therefore capacitance may be of greater use
(Firstenberg-Eden & Zindulis, 1984). Although all

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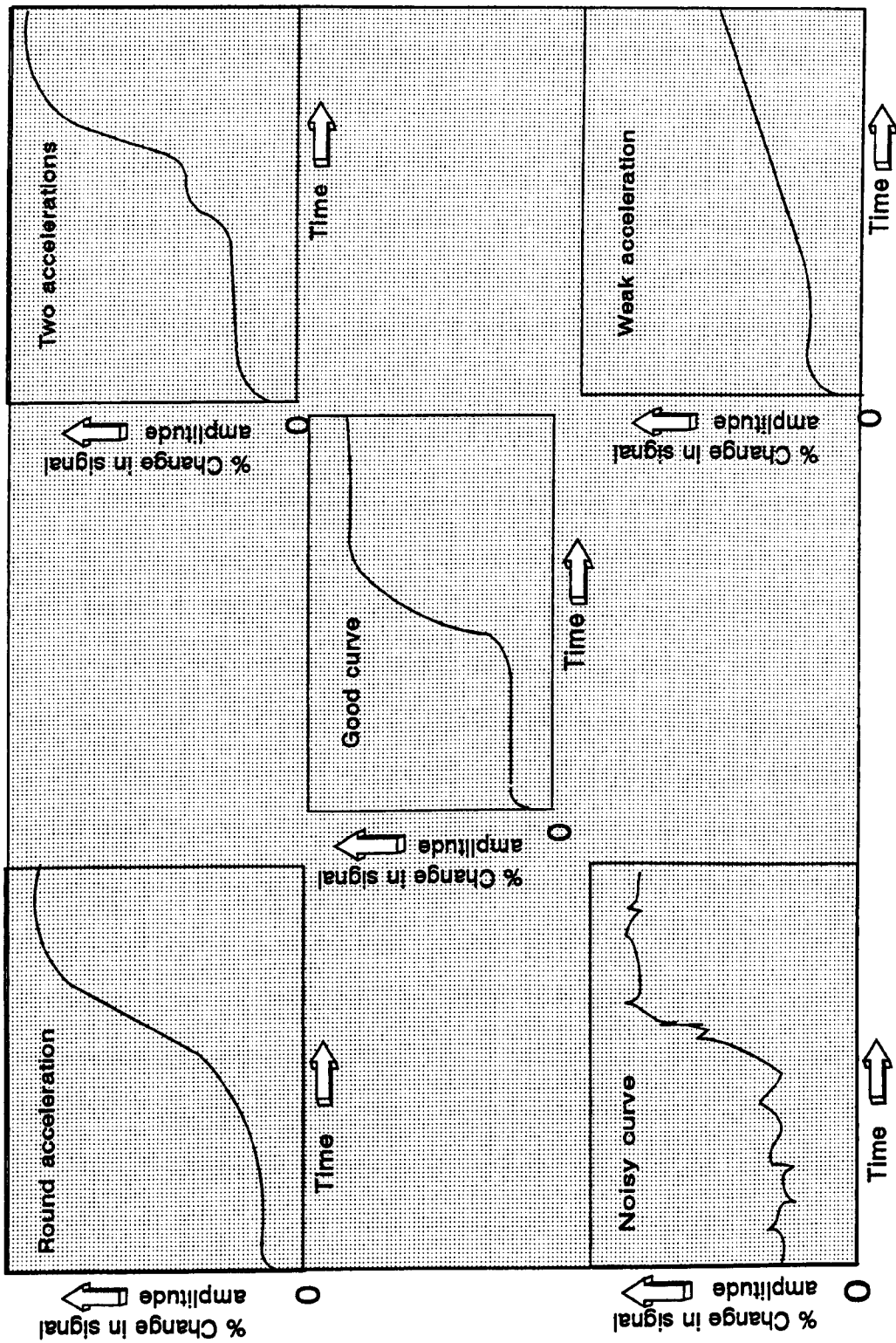
Fig. 3.3 Comparison of an electrical curve displaying good morphological characteristics with others with characteristics which are difficult to interpret.

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monitorable electrical algorithms are interrelated (Silley, 1991), the optimal algorithm requires careful evaluation, and will depend upon the target species and the composition and relative conductivity of the medium.

The sources of the electrochemical changes that result from microbial metabolism in complex culture media have been described by Owens (1985). Metabolic activities involve primarily the breakdown of polymers. Carbohydrate hydrolysis yields uncharged sugar monomers and therefore conductivity changes will depend on the extent of metabolism of these monomers. The proteins and peptides which occur in microbiological media carry only a small net charge at the normal range of pH values used (4 to 8), and, as the molecules are large, contribute little to conductivity. Hydrolysis of proteins to amino acids probably only causes a slight increase in conductivity due to the formation of zwitterions at these pH levels. Furthermore, any charged amino acids will be utilised by rapidly growing cultures, preventing accumulation. Oxidation of amino acids by aerobic organisms, however, leads to the excretion of ammonium (NH_4^+) ions and a subsequent large increase in conductivity. This is even greater in organisms capable of anaerobic fermentation, such as Clostridium spp., as high yields of organic acids as well as NH_4^+ ions are produced. Hydrolysis of triglyceride fats to free fatty acids and glycerol increases conductivity due to the production of protons. Inorganic nutrients, for example salts, utilised by cells during growth are present only in small amounts and

therefore have little effect on the overall conductivity of a medium. On the other hand the production of carbon dioxide (CO_2) in an impedimetric medium can have a number of outcomes. The CO_2 can be released to the atmosphere, it can remain in solution as dissolved CO_2 or it can form carbonic acid. The former two outcomes have little effect on proton flux and therefore conductivity whereas the latter affects this parameter (Owens, 1985).

With regards to development of an impedimetric medium for the facultative anaerobe L. monocytogenes, it can be predicted that the organism will be capable of producing protons, most importantly mobile NH_4^+ ions, and organic acids from nutrient mixtures containing complex carbohydrates, proteins and fats. The well known undefined bacterial growth medium nutrient broth provides such a nutritional environment. As glucose, which is known to be a growth enhancing substrate of L. monocytogenes (Kramer & Jones, 1969), produces lactic acid on metabolism which ionises to charged particles (Jones, 1989), this sugar could be a useful medium component. Nutrient broth supplemented with glucose (that is GNB) was therefore chosen as the basal medium for evaluation.

3.2 Results

The objective of the experiments described in this chapter was to evaluate the suitability of GNB as an impedimetric broth for the rapid detection of L. monocytogenes. The aim

of the first experiment was to find the optimal electrical algorithm for monitoring L. monocytogenes (Table 3.1, Fig. 3.4). A series of experiments was then carried out to determine whether GNB allowed L. monocytogenes to produce electrical curves of adequate quality and reproducibility in both morphology and amplitude in comparison with other test microorganisms (Tables 3.2 & 3.3, Fig. 3.5; 3.6). In addition, the threshold limits of detection of L. monocytogenes in GNB were examined (Table 3.4, Fig. 3.7; 3.8). Initially the reported optimal physical conditions for L. monocytogenes were used for all assays, that is a pH of 7.2, a temperature of 30 °C (Kramer & Jones, 1969; Leighton, 1985) and a sodium chloride concentration of 5 g dm^{-3} (Kramer & Jones, 1969). Latter experiments were then conducted to evaluate the optimal pH (Table 3.5) and sodium chloride concentration (Table 3.6, Fig. 3.9) under impedimetric conditions.

3.2.1 Comparison of the available electrical algorithms for detection of L. monocytogenes

To determine the optimal electrical algorithm for monitoring the target species, L. monocytogenes NCTC 11994, grown under the routine conditions described in the methods (see 2.3), was inoculated into GNB and the impedance, conductance and capacitance signals were measured for 36 h (for general assay regimen see 2.6). DT's and magnitudes of electrical signals were determined (see 2.6.1). The data obtained are reported in Table 3.1

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and shown diagrammatically in Fig. 3.4.

From Table 3.1 it can be seen that using capacitance and impedance curves resulted in similar DT's, while conductance curves produced longer DT's. Using the students t-Test (see 2.14) it was determined that there was no significant difference between impedance and capacitance DT's even at the 20 % level (t = 0.239). However impedance DT's were significantly different from

	Impedance DT magnitude (h) (BUIC)		Conductance DT magnitude (h) (BUCO)		Capacitance DT magnitude (h) (BUCA)	
Mean	6.71	1066	7.95	336	6.63	1982
SD	0.21	122	0.82	25	0.74	388

(N=6)

Table 3.1 Electrical responses of L. monocytogenes NCTC 11994 in GNB monitored simultaneously by impedance, conductance and capacitance.
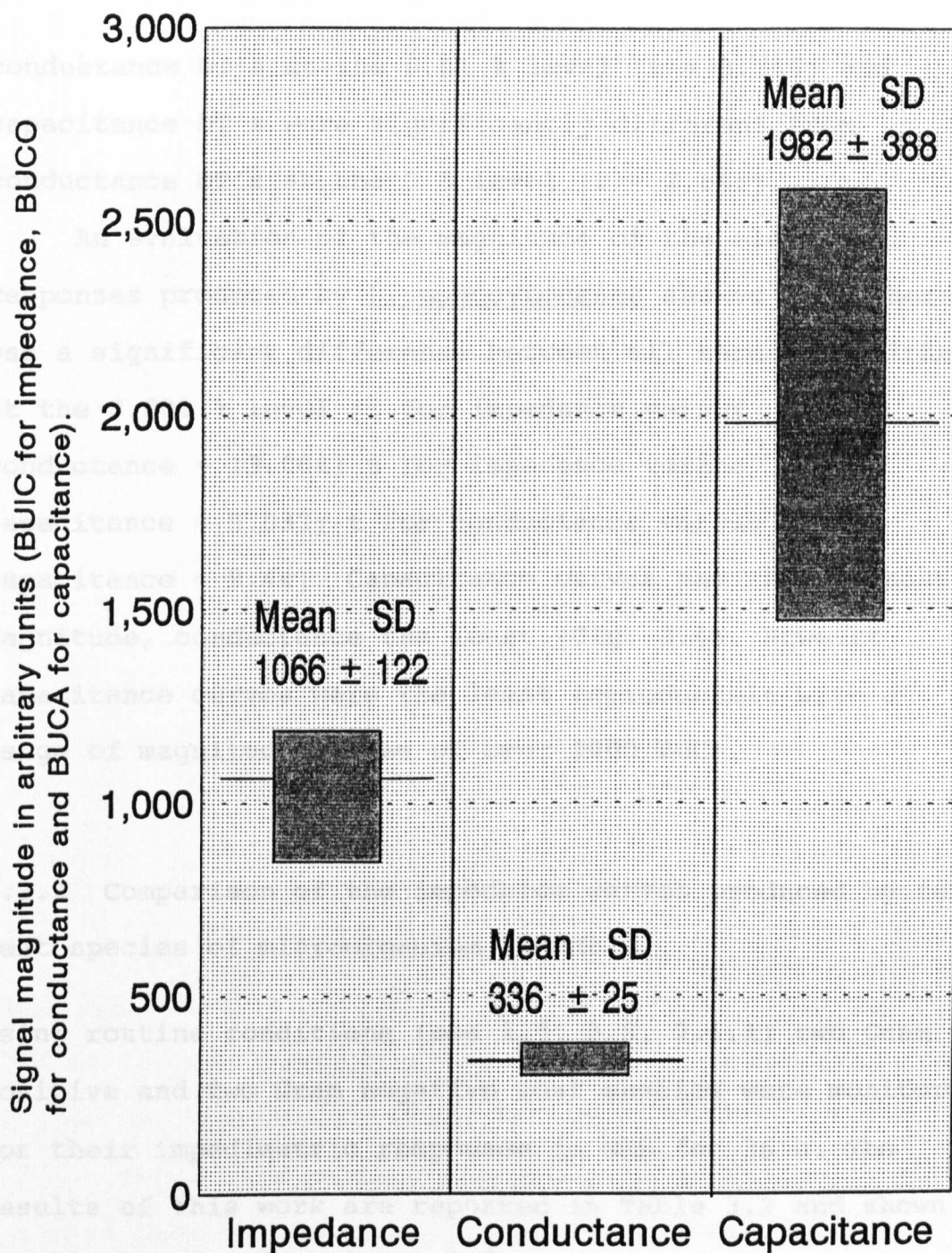
Figure 3.4 The mean, range and SD of the magnitude of the impedance, conductance and capcitance signals produced by L. monocytogenes NCTC 11994 in GNB. In each case the dark grey shading represents the range, the horizontal line the mean and the SD is given as a figure.

conductance DT's at the 0.01 % level (t = 3.346) and capacitance DT's were significantly different from conductance DT's at the 5 % level (t = 2.646).

An evaluation of the magnitude of the electrical responses produced by L. monocytogenes showed that there was a significant difference between all three algorithms at the 0.001 % level (t for impedance versus conductance = 13.004; t for impedance versus capacitance = 5.032; t for conductance versus capacitance = 9.44). Capacitance curves had the greatest magnitude, conductance the least (Fig. 3.4). However, capacitance curves were the least reproducible with a range of magnitude values of over 1000 BUCA.

3.2.2 Comparison of the impedance curves produced by four test species of microorganism in GNB

Using routine conditions (see 2.3, 2.6, 2.6.1) two Gram positive and two Gram negative test species were monitored for their impedimetric responses in GNB for 36 h. The results of this work are reported in Table 3.2 and shown graphically in Figs. 3.5 & 3.6.

The morphology of the impedance curves was found to vary between the species. As can be seen in Fig. 3.5, there were no shoulders to the curves produced by the Gram

Test species	Impedance magnitude MEAN ± SD
L. monocytogenes NCTC 11994	1090 ± 174
Lact. lactis NCIB 6681	767 ± 138
Ps. fluorescens NCIB 3756	808 ± 147
E. coli NCIB 86	814 ± 225

(N=15)

Table 3.2 The mean impedance magnitudes of four test species in GNB.

negative organisms, Ps. fluorescens and E. coli, while the two Gram positive species, L. monocytogenes and Lact. lactis, produced well defined shoulders. Of the four species tested, L. monocytogenes produced impedance curves of greatest magnitude and morphological quality (Fig. 3.5 & 3.6). Use of the students t-Test (see 2.14) showed that the magnitude of the impedance curves produced by L. monocytogenes was significantly higher than those of the three other test species (Table 3.3). Even at the 20 % level there were no significant differences in the magnitude of electrical responses produced by the other three test species.

Fig. 3.5 Typical impedimetric responses of four test species in GNB.

L. monocytogenes
NCTC 11994

Lact. lactis
NCIB 6681

Ps. fluorescens
NCIB 3756

E. coli
NCIB 86

Time

24 h

Reciprocal of % change in impedance
amplitude

0
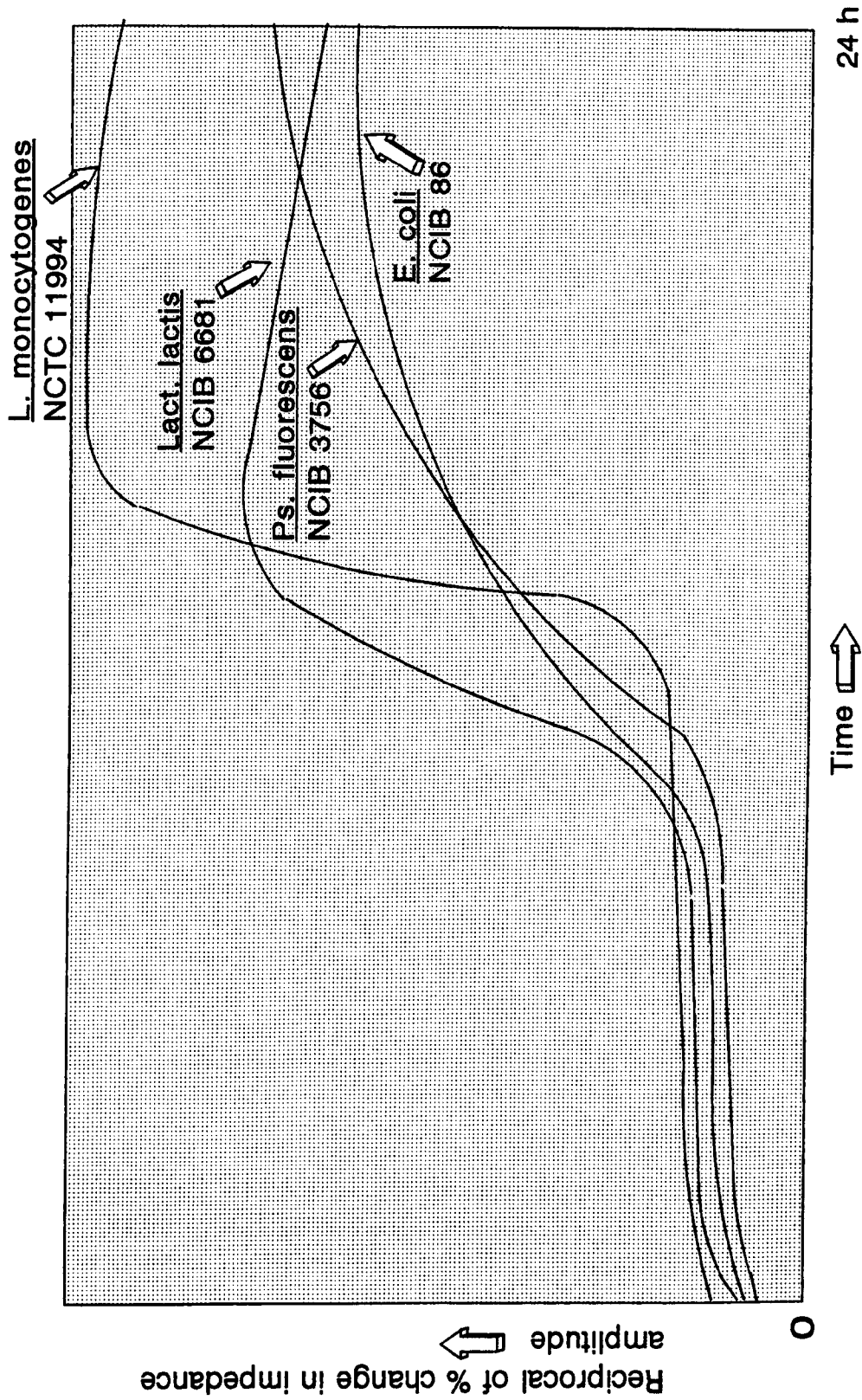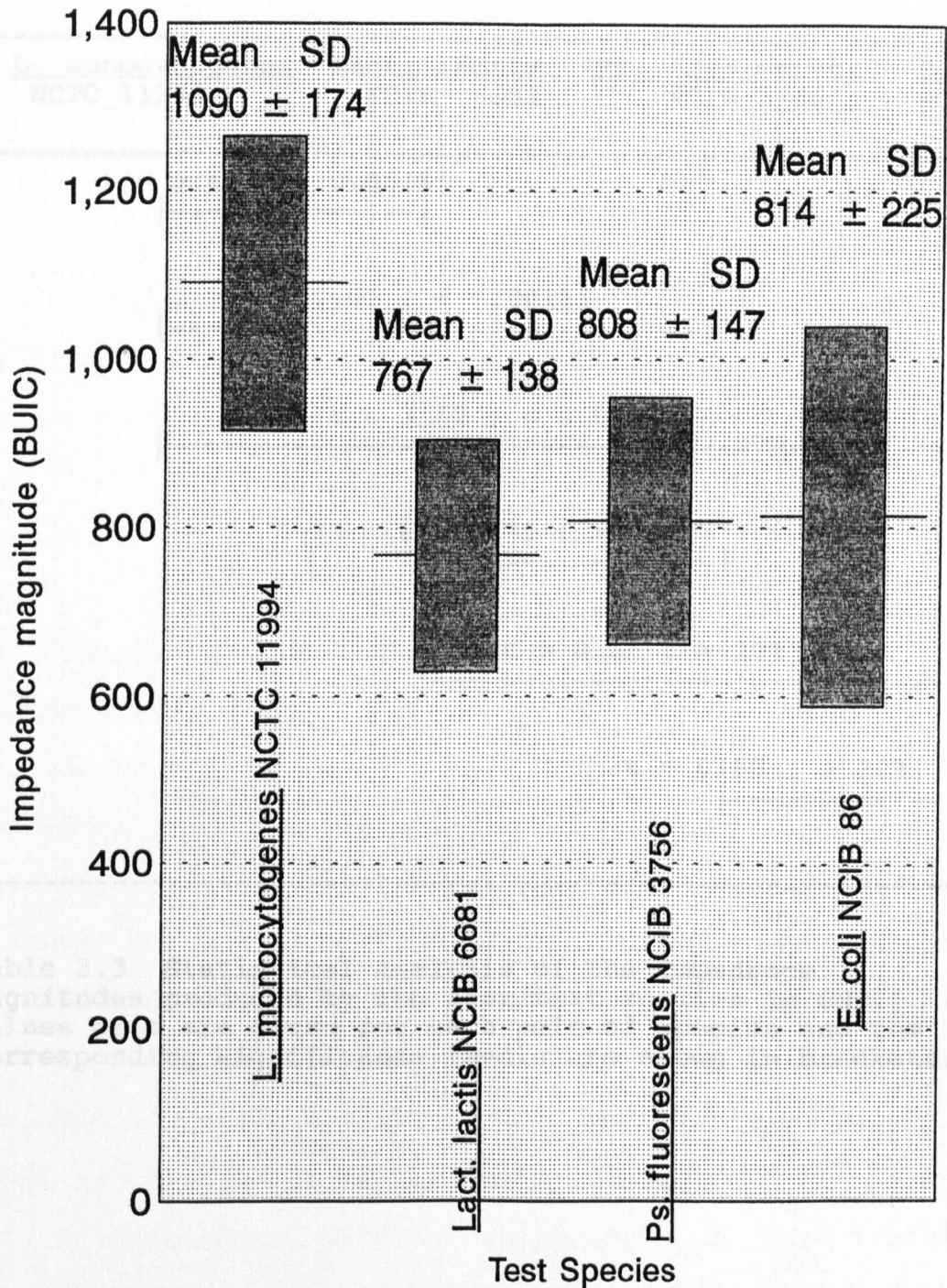
Figure 3.6 The mean, range and SD of the magnitude of the impedimetric signal produced by four test species in GNB. In each case the dark grey shading represents the range, the horizontal line the mean and the SD is given as a figure.

```
/------------------------------------------------------------------\
|  L. monocytogenes    Lact. lactis    Ps. fluorescens    E. coli  |
|     NCTC 11994        NCIB  6681       NCIB 3756        NCIB 86   |
|                                                                  |
|------------------------------------------------------------------|
|       t = 5.42 ( < 0.001%)                                       |
|        |-------------|                                           |
|                                                                  |
|                                                                  |
|            t = 4.62 ( < 0.001%)                                  |
|        |----------------------------|                            |
|                                                                  |
|                                                                  |
|               t = 3.62 ( < 0.02%)                                |
|        |-----------------------------------------|               |
|                                                                  |
|                                                                  |
|                 t = 0.75 ( > 20%)                                |
|                 |----------------|                               |
|                                                                  |
|                                                                  |
|                    t = 0.66 ( > 20%)                             |
|                 |----------------------------|                   |
|                                                                  |
|                                                                  |
|                       t = 0.08 ( > 20%)                          |
|                                 |------------|                   |
|                                                                  |
|                                                                  |
\------------------------------------------------------------------/
```

Table 3.3 Statistical analysis of the impedance
magnitudes produced by the four test species in GNB.
Values of t are given for each pair of species and the
corresponding significance levels are shown in brackets.

3.2.3 Determination of the limits of detection of

L. monocytogenes by impedimetric assay

To assess the limits of detection, L. monocytogenes
NCTC 11994 was grown under routine conditions (see 2.3),
and then 0.1 cm^3 samples of a range of dilutions (10^{-1} to
10^{-8}) were simultaneously evaluated for TVC (see 2.4) and
impedimetrically monitored for 36 h in 1 cm^3 of GNB (see
2.6, 2.6.1 & 2.6.2). The results obtained are both
tabulated (Table 3.4) and shown graphically (Fig. 3.7 &
3.8).

Impedance curves of high magnitude and
reproducibility were produced throughout the range of
inocula tested, enabling easy identification of the DT and
therefore potential enumeration of L. monocytogenes (Table
3.4, Fig. 3.7). A negative correlation (r = - 0.9954) was
observed between DT and TVC (Fig. 3.8). This was found to
be significant at the 0.1 % level. Of major importance is
the observation that the impedimetric assay regimen used
was able to detect low TVC's of L. monocytogenes such as
might be expected to be present in foodstuffs.

In preliminary experiments problems of detection had
been encountered with high cell concentrations. As initial
temperature fluctuations can have a significant effect on
the morphology of electrical curves, the Bactometer
automated software does not register impedance responses
initiated in periods up to 1 h after monitoring commences.
To overcome the problem of curves being produced within
this time period, a 10^{-4} dilution was routinely used as

TVC (CFU cm^{-3}) $\pm$ SD	Impedance	
	DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
$1.18 \pm 0.12 \times 10^8$	0.9 ± 0.09	1041 ± 161
$1.18 \pm 0.12 \times 10^7$	2.9 ± 0.09	1163 ± 206
$1.18 \pm 0.12 \times 10^6$	4.9 ± 0.21	956 ± 203
$1.18 \pm 0.12 \times 10^5$	7.0 ± 0.30	1371 ± 469
$1.18 \pm 0.12 \times 10^4$	10.2 ± 0.29	1253 ± 359
$1.18 \pm 0.12 \times 10^3$	12.5 ± 0.41	1057 ± 220
$1.18 \pm 0.12 \times 10^2$	14.8 ± 0.57	925 ± 286
$1.18 \pm 0.12 \times 10^1$	16.5 ± 0.48	1240 ± 384

(N=4)

Table 3.4 Comparison of TVC and impedimetric responses of L. monocytogenes NCTC 11994 in GNB

inoculum in later experiments. This dilution of

L. monocytogenes produced a DT of approximately 10 h

(Table 3.4).

3.2.4 Evaluation of the effect of pH on the impedimetric

detection of L. monocytogenes

The pH stability of GNB during preparation was first

determined. The pH of freshly prepared non autoclaved, non

pH adjusted GNB was 6.36. The medium was then adjusted to

pH 7.2 (the optimum for the target species) by the

addition of 0.1 M sodium hydroxide and sterilised by

autoclaving. After cooling to room temperature, the pH was

again determined and found to be 7.13. Due to this
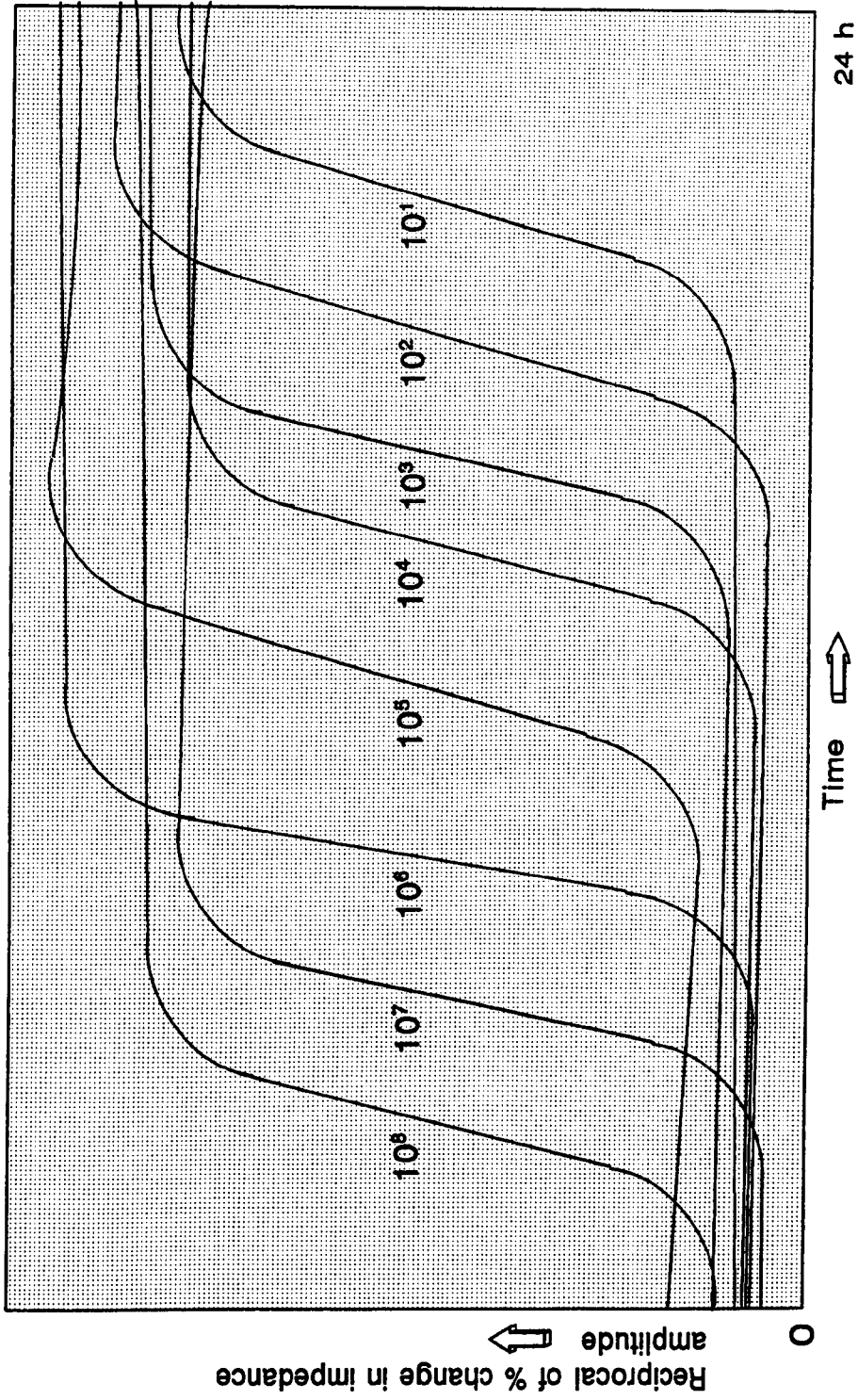
Fig. 3.7 Typical impedimetric responses of a range of inoculum concentrations of *L. monocytogenes* NCTC 11994 in GNB. Values shown on graphs are in CFU cm^{-3}.
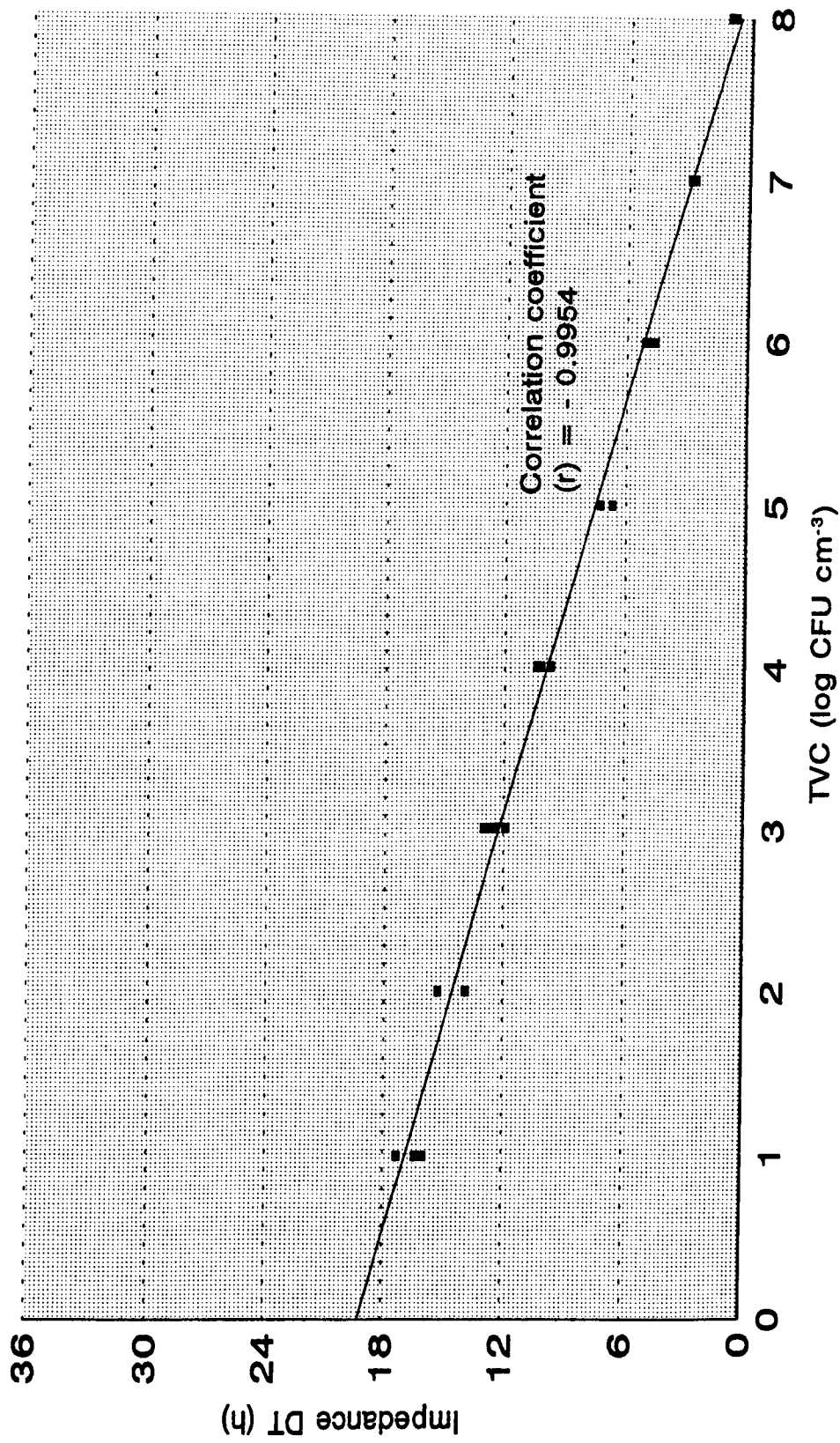
Fig. 3.8 Relationship between impedance DT and TVC in L. monocytogenes NCTC 11994.

Correlation coefficient (r) = - 0.9954

TVC (log CFU cm^{-3})

Impedance DT (h)

consistent 0.07 pH unit reduction of GNB during
autoclaving, in later experiments the pH was always
adjusted 0.07 units above the desired value prior to
autoclaving.

GNB was adjusted to a range of values above and below
pH 7.2 the reported optimum for L. monocytogenes (Kramer &
Jones, 1969) by the addition of 0.1 M sodium hydroxide
(BDH Biochemicals) or 0.1 M hydrochloric acid (BDH
Biochemicals). Using routine conditions (see 2.6 & 2.6.1)
the impedimetric responses of L. monocytogenes NCTC 11994
were then monitored for 36 h in the modified medium. The
results are shown in Table 3.5.

L. monocytogenes was observed to be able to produce
an impedimetric response at all pH values examined, with
pH 7.2 being optimal for rapidity of detection. The pH
range of 7.2 to 8.2 produced impedance curves of greatest
magnitude.

pH	DT (h) $\pm$ SD	Impedance magnitude (BUIC) $\pm$ SD
5.2	15.6 $\pm$ 0.15	754 $\pm$ 16
6.2	10.4 $\pm$ 0.75	926 $\pm$ 30
7.2	9.6 $\pm$ 0.52	988 $\pm$ 81
8.2	12.2 $\pm$ 0.05	1061 $\pm$ 34
9.2	13.3 $\pm$ 0.05	877 $\pm$ 11

(N=2)

Table 3.5 The effect of pH on the impedimetric responses
of L. monocytogenes NCTC 11994.

3.2.5 Evaluation of the effect of sodium chloride concentration on the impedimetric responses of four test species

The influence of the concentration of sodium chloride in GNB on the impedimetric responses of four test species was examined (see 2.3, 2.6 and 2.6.1). The results obtained are given in Table 3.6 and Fig. 3.9.

Test species	Sodium chloride (g dm^{-3})	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
L. monocytogenes NCTC 11994	2	9.8$\pm$0.15	713$\pm$ 3
	5	9.8$\pm$0.05	1135$\pm$31
	20	10.5$\pm$0.19	2750$\pm$52
	45	13.1$\pm$0.15	3366$\pm$77
Lact. lactis NCIB 6681	2	7.9$\pm$0.15	475$\pm$ 3
	5	7.7$\pm$0.10	621$\pm$ 6
	20	8.3$\pm$0.05	1059$\pm$25
	45	16.6 #	943
Ps. fluorescens NCIB 3756	2	9.2$\pm$0.35	352$\pm$ 3
	5	9.1$\pm$0.30	396$\pm$76
	20	13.6$\pm$0.65	409$\pm$96
	45	> 36 h	0
E. coli NCIB 86	2	7.9$\pm$0.30	446$\pm$ 8
	5	7.4$\pm$0.35	630$\pm$20
	20	11.1 #	1057
	45	28.2 #	1040

(N=2)

Key
= One artefactual curve produced.

Table 3.6 The effect of sodium chloride concentration on the impedimetric responses of four test species.
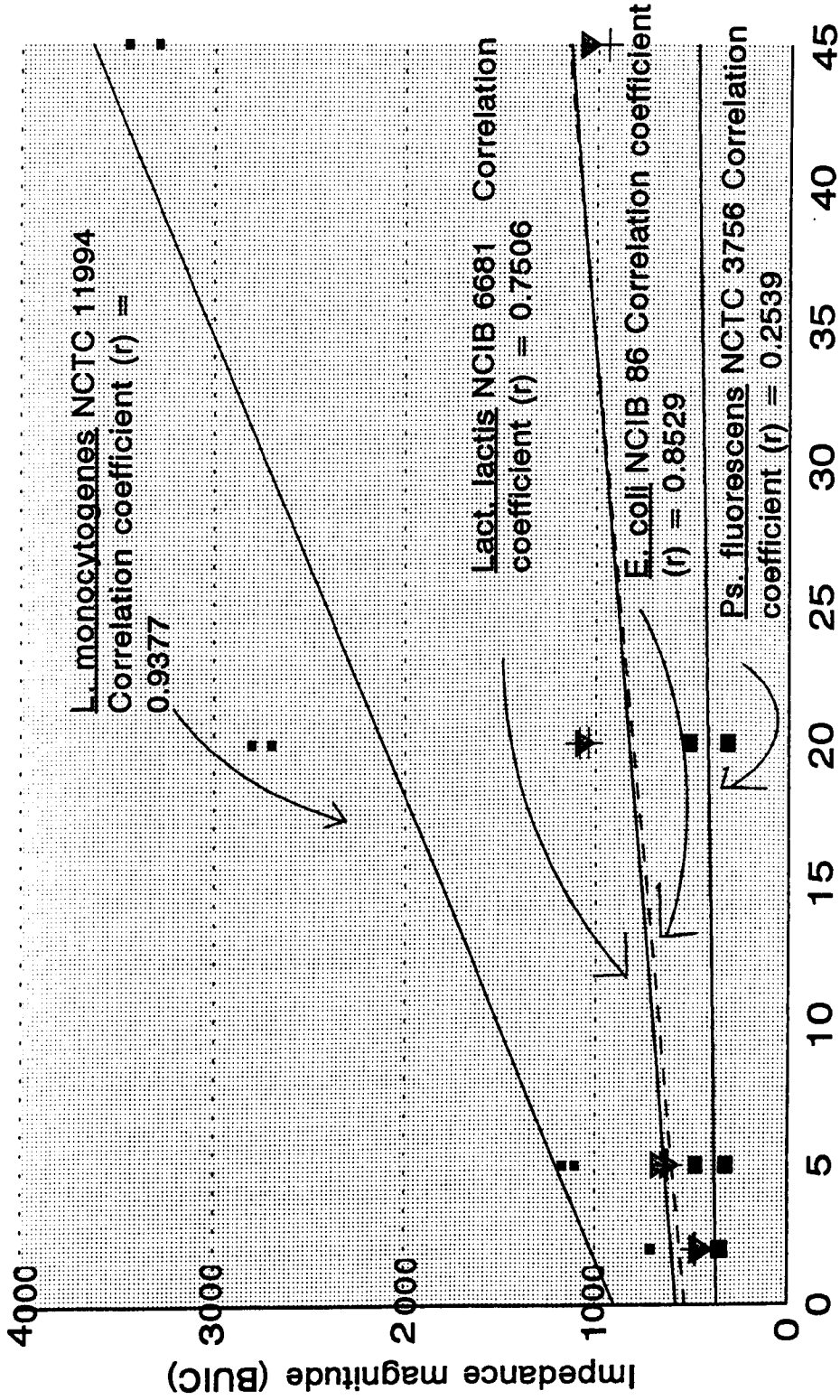
Fig. 3.9 Effect of sodium chloride concentration on the impedance magnitude of four test species.

L. monocytogenes produced a DT at all concentrations of sodium chloride examined, although the DT started to increase above concentrations of 5 g dm^{-3}. Similar increases in the DT's of the other three test species were observed (Table 3.6). The impedance magnitude of all four test species examined also increased with increasing concentration of sodium chloride (Fig. 3.9). Statistical analysis (see 2.14) showed that there was a significant correlation at the 0.1 % level between the impedance magnitude produced by _L. monocytogenes_ and the concentration of sodium chloride in the medium. In _E. coli_ correlation was significant at the 5 % level and in _Lact. lactis_ at the 10 % level. However, there was no significant correlation between impedance magnitude and sodium chloride concentration in _Ps. fluorescens_. Furthermore, the latter test species was selectively inhibited by the highest concentration (45 g dm^{-3}) of sodium chloride used.

In this series of experiments a number of curves with unusual features were produced (see Fig. 3.10 for examples). As it was impossible to determine the DT and impedance magnitude from these artefactual curves and they were easily differentiated from electrical curves with normal characteristics, they were simply discarded. Such curves could have resulted from electrode interference, for example gas production reducing the surface area of the electrodes.
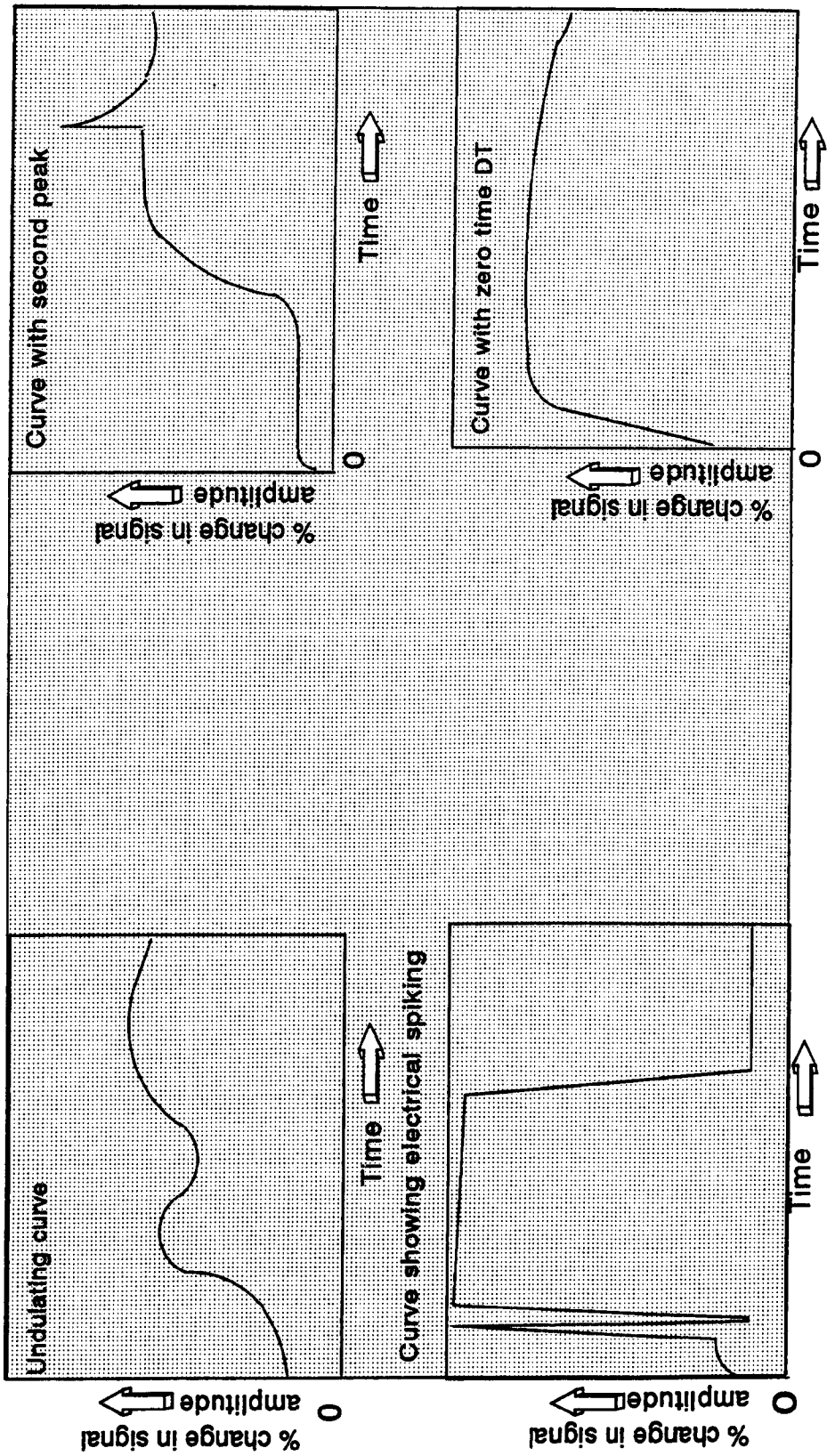
Fig. 3.10 Illustration of a variety of types of artefactual curve.

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3.3 Discussion

Owens (1985) commented that the selection of the constituents of an impedimetric medium is an empirical trial and error procedure which is expensive in time and effort. This can be confirmed by the current work.

Much thought was given to the choice of test species for examination. L. monocytogenes NCTC 11994 was chosen for routine screening as it is a typical haemolytic strain, whereas the type strain L. monocytogenes NCTC 10357, being non haemolytic, is atypical of the species. As the type strain was isolated in 1924 (Murray et al., 1926), much time has elapsed during which its original characteristics could have changed in laboratory culture. The other three test species, Lact. lactis, Ps. fluorescens and E. coli, were chosen as representative Gram positive and negative organisms.

Stock cultures of the test species were routinely grown in nutrient broth to prevent "carry over" of nutrients not present in the impedimetric medium into the Bactometer reaction wells. As it was intended to develop the enumeration applications of the assay, the choice of diluent was also carefully considered. To limit microbial replication a non-nutrient based diluent, 1/4 strength Ringers solution, was used in preference to a nutrient based diluent, such as peptone water. A 0.1 cm^3 inoculum of test organism as used by Arnott et al. (1988) was found to be satisfactory.

Impedance was the algorithm chosen for initial work

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as L. monocytogenes produced very poor quality conductance curves in GNB (Table 3.1, Fig. 3.4) and capacitance has been reported to be insensitive for detecting bacterial growth (Richards et al., 1978). Although in this work capacitance was found to give electrical curves of the greatest magnitude, that is it was the most sensitive signal, it was also the most variable in response. Furthermore, as the capacitance signal is principally electrode based, in later work (see Chapter 5) it could be particularly sensitive to the deposition of food material onto the electrodes, thereby increasing the proportion of artefactual curves produced such as those shown in Fig. 3.10. Due to these inherent problems with capacitance, the lack of knowledge of this electrical algorithm (Owens, 1985), and the fact it did not yield a significant reduction in DT, it was not considered an appropriate algorithm for use in this work. On the other hand impedance, which is related to both conductance and capacitance (Firstenberg-Eden & Zindulis, 1984; Silley, 1991), produced good quality curves under the routine experimental conditions used.

The basal impedimetric medium chosen for evaluation was Nutrient Broth No. 2. This medium was identified as being particularly useful for the growth of Listeria by Ralovich (1984). It was also used by Kramer & Jones (1969) for the growth of L. monocytogenes in selective conditions and by Dominguez et al. (1984) to provide essential nutrients in an enrichment medium. The medium was supplemented with glucose, the sugar which is considered

the best energy source for <u>Listeria</u> spp. (Seeliger &
Jones, 1986; Lachia, 1990). This allowed <u>L. monocytogenes</u>
to produce impedance curves of greater magnitude than the
other three test species examined (Fig. 3.5 & 3.6). The
magnitude of the response is of major importance as this
parameter increases the sensitivity of an electrochemical
assay (Hogg <u>et al</u>., 1987).

The observed difference in impedance magnitude
between <u>L. monocytogenes</u> and the other test species could
be further increased to differentiate the target organism
in a qualitative type assay. To maximise electrical
changes Owens (1985) suggested criteria in the following
order were important: buffer pH, proton flux, medium
constituents (especially the electron donor, electron
acceptor, carbon source and nitrogen source) and metabolic
products. Both Firstenberg-Eden & Zindulis (1984) and
Owens (1985) stated that although a reduction in pH is not
the main cause of the conductivity signal, protons do
produce large changes in capacitance (see 1.5.1) and
therefore impedance.

To enhance impedance magnitude, medium constituents
should be used which are involved in as many metabolic
processes as possible that produce or consume protons.
These constituents should be coupled with a buffer that
magnifies proton flux by exhibiting a large change in
conductivity on the acquisition or loss of protons. As the
proton flux determines the magnitude of the
buffer-mediated pH change, nutrients should be chosen
which promote the maximum flux of protons in one

direction, that is, "lock" the organism into a particular type of metabolism (Owens, 1985). In addition the buffer should retain the pH at or near the optimum for the target organism to ensure rapid metabolism and therefore rapid detection.

There are a number of problems associated with the development of a qualitative type assay. To channel an organism into a particular metabolism requires a narrow spectrum of nutrients, however the speed of impedimetric detection will be reduced if only a limited range of amino acids, carbohydrates and fats are supplied. Furthermore, as the eventual aim of this work is the detection of L. monocytogenes in foods, the influence of food components must be considered. Due to its complicated chemical nature, the presence of a food matrix would inevitably alter the nutrient spectrum supplied and hence preclude metabolic channelling. As the qualitative type of assay requires monocultures of the target organism, the mixed microflora of foods would alter the morphological characteristics of the detection curves produced. Any methods used to obtain a monoculture of the target organism prior to a qualitative impedimetric assay would be time consuming, labour intensive and defeat the object of rapid detection. Furthermore they would not remove the exoenzymes produced by microorganisms in certain foods which Owens (1985) reported could affect conductivity. In addition to the complication of differences in substrates and microflora, Bolton (1990) described an inter-experiment variation in electrical signal with one

medium and one test species. It was noticed that impedance magnitude varied with different batches of the basal medium. This could be due to slight variations in time of autoclaving, in contamination levels on glassware or in the surface area of the electrodes. Firstenberg-Eden (1984), for example, has recorded a decrease in capacitance with reuse of electrodes.

In an ideal quantitative assay the target organism should be detected rapidly, producing electrical curves of high quality, magnitude and reproducibility. The DT should be determined by a sharp acceleration enabling reliable cell enumeration. No detection curves should be produced by any non target species present. Unsupplemented GNB (that is GNB without any additional selective agents) did not allow the quantitative detection of L. monocytogenes (Table 3.2). Despite this, the quantitative type assay holds more promise than the qualitative type assay for future application to foods. In the former assay there is only a single morphological prerequisite for identification (that is the presence or absence of a recorded DT within the monitoring period) while the latter assay requires expert interpretation of electrical curves.

There are a number of potential ways of improving the quality of electrical curves. For example, as shown in this work, by an increase in concentration of sodium chloride (Table 3.6). Hogg et al. (1987) suggested adding agents to increase the conductivity changes exhibited by electrical media. Owens (1985) outlined other ways of improving electrical responses: Firstly, reduction of ion

pair formation with inorganic nutrients by the use of uni-univalent salts, such as sodium chloride, whenever possible in preference to uni-bivalent salts, such as calcium chloride. Secondly, alteration in the protein content of the medium to include proteins that are metabolised at the pH range 4 to 8 and that contain high concentrations of amino acids like cysteine carrying charge at these pH values. Thirdly, use of polypeptides or amino acids with high nitrogen to carbon ratios to enable greater production of NH_4^+ ions. As an alternative if the reaction wells were covered with sterile oil, attempts could be made to utilise bicarbonate formation from CO_2 to improve the quality of the electrical responses. CO_2 itself is known to stimulate the growth of L. monocytogenes (Skovgaard, 1989). Unfortunately, trapping the produced CO_2 may create gas bubbles on the electrodes thus reducing surface area and decreasing the quality of curves (Connolly et al., 1988).

In developing a quantitative type assay the objective is to achieve a DT for low numbers of target organisms which is less than the time required for detection by traditional methodology. Higher concentrations of target organisms are detected even more rapidly. The lower limit of detection of L. monocytogenes by impedimetric assay in GNB was approximately 10 cells detected in under 17 h (Table 3.4, Figs. 3.7 & 3.8). A significant relationship was found to exist between impedance DT and inoculum concentration (Fig. 3.8). This is as expected; many workers have reported a good correlation between DT and

CFU (Firstenberg-Eden, 1983; Firstenberg-Eden et al.,
1984; Shapton & Cooper, 1984; van Spreekens &
Stekelenberg, 1986; Nieuwenhof & Hoolwerf, 1987). GNB
therefore permitted rapid impedimetric detection of low
levels of L. monocytogenes. To improve the assay, growth
enhancing factors, for example glutamine and ferric
chloride (Kramer & Jones, 1969; Leighton, 1985), could be
added to increase metabolic activity and hence reduce
DT's.

A time limit is set in quantitative type assays to
enable the differentiation and rejection of late detection
curves produced by non target organisms. The DT of 17 h
for 1 to 10 CFU of L. monocytogenes (Table 3.4) suggests
an appropriate cut off time after which any detection
would be the result of a non Listeria species. To increase
the differentiation between Listeria and non-Listeria
organisms selective agents or antibiotics can be added to
the medium to suppress the growth and electrical signals
of the latter (Arnott et al., 1988; Connolly et al.,
1988). Taking into account the expected partial inhibition
of L. monocytogenes by such agents and the fact that the
assay is planned for future development with foods, which
themselves often have intrinsic antimicrobial properties,
a cut off time of more than 24 h might be required to
detect low levels of Listeria. Cells damaged as a result
of food processing techniques may metabolise more slowly
than normal and hence have delayed DT's. As the test
species were grown in GNB prior to their impedimetric
monitoring in GNB in these preliminary experiments, it is

likely that DT's were reduced as a result of the induction of any necessary enzymes during the former culture. Organisms from foods would have experienced a different nutritional environment before impedimetric monitoring and hence DT's may be increased by the requirement for enzyme induction in the new medium. Due to these factors a self-imposed time limit for detection was initially set at 36 h.

To prevent high cell concentrations of L. monocytogenes producing a detection within the temperature stabilisation period of the Bactometer, dilution of samples prior to their inoculation into the reaction wells was used as a simple precautionary measure. This could be beneficial in food samples as it could decrease the concentration of preservatives present and reduce deposition of food materials on electrodes. In practice, however, the stabilisation period prior to monitoring should not be a problem as only low levels of L. monocytogenes would be expected to be present in foods. Cady et al. (1978) and Firstenberg-Eden & Zindulis (1984) discussed the necessity for an initial equilibration period of 0.5 to 1.5 h. They stated this was required for bulk medium conductance and polarisation capacitance to equilibrate as both are affected by temperature. Conductance was reported to have a temperature coefficient of 1.5 to 2 % per °C depending on the thermal time constant of the test cell and the initial temperature difference. The time to reach capacitance equilibration depended on electrochemical equilibration, viz the time

required for the dielectric to form, and had a temperature constant of 1 % per °C. Monitoring electrical changes during the time when the temperature is stabilising would obviously affect the morphology of the detection curves and could result in a premature DT.

From the investigation of the effect of pH on the impedimetric detection of L. monocytogenes (Table 3.5) it was noted that at 7.2, the optimal pH for L. monocytogenes (Kramer & Jones, 1969; Leighton, 1985), the target species produced its shortest DT. In addition L. monocytogenes was found to produce an impedance curve at a wide range of pH values. This could be exploited in a quantitative type assay for the selective elimination of non-target species which are not as tolerant to extremes of pH.

The investigation into the effect of sodium chloride concentration (Table 3.6, Fig. 3.9) revealed that the level present in basal GNB, 5 g dm^{-3} (Kramer & Jones, 1969), was optimal of the concentrations tested for the rapid impedimetric detection of L. monocytogenes. It has been reported that L. monocytogenes is very tolerant to sodium chloride compared with other microorganisms (Farber, 1989b; Farber et al., 1992). For example while L. monocytogenes is capable of growth in 130 to 140 g dm^{-3} of sodium chloride and capable of surviving 200 g dm^{-3}, one of the other test species Ps. fluorescens is inhibited at 45 g dm^{-3}. High salt concentrations could therefore be used for the selective inhibition of non Listeria species.

During the work on sodium chloride concentration a number of electrical signals of uncharacteristic

morphology were produced (Fig. 3.10 for examples of artefactual curves). Cossar et al. (1990) reported that such artefactual data are nearly always due to electrode disturbances and much research is now taking place into these responses.

In conclusion it was decided that GNB is suitable for both the growth of L. monocytogenes and the active production of electrolytic products of metabolism. However, GNB requires modification to enable the rapid selective detection of L. monocytogenes by a quantitative type assay. The evaluation of antimicrobial agents selective for L. monocytogenes is described in Chapter 4 and the application of the selective impedimetric assay to foodstuffs is considered in Chapter 5.

CHAPTER 4

EVALUATION OF ANTIMICROBIAL AGENTS FOR USE IN A SELECTIVE IMPEDIMETRIC ASSAY FOR L. monocytogenes

4.1 Introduction

It is possible to detect individual bacterial species or related groups of species by electrically monitoring growth medium containing selective antimicrobial agents (Hadley & Yajko, 1985). The purpose of the antimicrobial agents is to suppress the metabolism of the non target species which include other bacteria, moulds and yeasts. The aim of the research described in this chapter was to determine one or more toxic agents that could facilitate the impedimetric selection of L. monocytogenes.

The basis of selective toxicity has been discussed by Albert (see for example Albert et al., 1945; Albert & Goldacre, 1948; Albert, 1965; Albert, 1985). He has defined "selectivity" as an influence on one type of living cell without corresponding affect on others, and "selective toxicity" as a strong effect on one type of cell without any effect on others, even when both types are growing side by side. Cells that are affected by an antimicrobial agent are called uneconomic cells, whilst those that remain unaffected are termed economic. Common examples of selective toxicity include pesticides employed in agriculture, such as insecticides used to kill insects

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without harming either crops or the consumer, and antibiotics used in medicine to eliminate pathogenic infections without injuring the mammalian host.

Toxicity depends upon the structure of the antagonist and its relationship with cellular receptors. Three arguments have been put forward in support of the concept of receptors: the high dilution at which agents can retain their potency; their biological specificity and the difference in effectiveness of optical isomers (Albert, 1985). A toxic agent must reach an appropriate receptor to elicit its toxic effects, and therefore access of the agent to receptor is crucial. The receptors for antimicrobial agents can be enzymic, for example penicillins block the transpeptidase enzymes bacteria require to form new cell wall. Other receptors are found on non catalytic macromolecules, for example acridine dyes, which are essentially flat molecules, intercalate between adjacent base pairs in DNA and induce frameshift mutations.

Three types of selectivity have been described by Albert (1965, 1985):

1. A biologically active agent may be equally toxic to all species but accumulate principally in the uneconomic species. This can be due to cytological or biochemical differences; for example, the uneconomic cells may have a greater surface area. This type of selectivity is therefore based on **comparative distribution.**

2. A biologically active agent may react with and injure a metabolite which is present in greater concentration in

the uneconomic species, or only present in the uneconomic species. This form of selectivity is therefore based on **comparative biochemistry.**

3. A biologically active agent may react exclusively with a cytological feature that only exists in the uneconomic species or existing in greater concentrations than in the economic species. This type of selectivity is therefore based on **comparative cytology.**

An impedimetric assay for the detection of L. monocytogenes would need to take advantage of one or more of the above principles of selectivity. For example, use might be made of the known cytological differences between the cell walls of Gram positive and Gram negative bacteria. The task of injuring or eliminating non listerial cells without damaging Listeria spp. has been practised in traditional selective microbiological methods for the isolation of L. monocytogenes. Selective agents found useful in these methods were evaluated in the present research for use in the selective impedimetric detection of L. monocytogenes. Initially, selective agents were evaluated for an ability to differentiate Gram positive from Gram negative and fungal cells. Potentially useful agents were then evaluated for their ability to differentiate the genus Listeria from other Gram positive species. The final objective was to determine one or more agents that allow the sole detection of L. monocytogenes and it was predicted that toxic agents that function on

the principle of comparative biochemistry might be the most effective for this purpose.

4.2   Results - Individual Antimicrobial Agents

A selection of antimicrobial agents, the majority of which had previously been used in media reported to be selective for <u>Listeria</u> spp., were evaluated for their spectrum of antimicrobial activity, minimum inhibitory concentrations and effects on the electrical responses of test microorganisms. The selective agents examined were nalidixic acid (see 4.2.1), thallous acetate (see 4.2.2), moxalactam (see 4.2.3), lithium chloride (see 4.2.4), phenylethanol (see 4.2.5), potassium tellurite (see 4.2.6), oxolinic acid (see 4.2.7), acriflavine hydrochloride (see 4.2.8) and proflavine hydrochloride (see 4.2.9).

Optimal cultural conditions, as determined in Chapter 3, were used throughout this work. For routine screening of selective agents a 10^{-4} dilution of an overnight culture was used as inoculum. In the absence of selective agents this concentration of the target species produced a DT of approximately 10 h (Table 3.4) and therefore avoids the complication of detection during the temperature equilibrium period of the Bactometer. As bactericidal or bacteriostatic activity would be expected to delay the DT, a quantitative estimation of the toxicity of individual antimicrobial agents was obtained by comparing the DT's recorded in supplemented and unsupplemented medium.

4.2.1 Investigation of nalidixic acid as an agent selective for L. monocytogenes.

Nalidixic acid has been described as being bactericidal to most of the common Gram negative species of bacteria, although pseudomonad species are resistant. It has been reported to be less active against Gram positive bacteria (Gilman et al., 1985).

Using routine conditions (see 2.3 & 2.5) the growth of four test species (chosen as representatives of different broad taxonomic groups of bacteria in terms of morphology and Gram staining characteristics) was determined spectrophotometrically in GNB supplemented with 0 to 60 mg dm^{-3} nalidixic acid. Nalidixic acid was added to the medium prior to autoclaving, as described by Kramer & Jones (1969) and Ralovich (1989). The results are tabulated in Table 4.1.

The growth of all four organisms was inhibited by nalidixic acid. However, as expected, the growth of the Gram negative microorganisms was affected to a greater extent than that of the Gram positive organisms. The growth of Listeria was the least affected.

Further work was undertaken to evaluate the effect of nalidixic acid on the electrical responses of the test microorganisms. Using routine conditions (see 2.3, 2.6 & 2.6.1) the impedimetric responses of the four test species individually and in mixed culture were monitored for 36 h in GNB supplemented with a range of concentrations of heat sterilised nalidixic acid. The impedance DT and magnitude

Test species	Concentration of nalidixic acid ($mg\ dm^{-3}$)	Absorbance at 610 nm after 24 h
L.	0	0.45
monocytogenes	20	0.44
NCTC 11994	40	0.37
	60	0.29
Lact.	0	1.10
lactis	20	1.00
NCIB 6681	40	0.80
	60	0.10
E. coli	0	0.35
NCIB 86	20	0.01
	40	0.01
	60	0.00
Ps.	0	0.66
fluorescens	20	0.06
NCIB 3756	40	0.02
	60	0.02

(N=1)

Table 4.1 Spectrophotometric assay of the effect of heat sterilised nalidixic acid on the growth of test species in GNB.

recorded at each concentration of nalidixic acid are given in Table 4.2.

The DT of L. monocytogenes was observed to increase with increasing concentration of heat sterilised nalidixic acid. For example there was a 6 h increase in DT as the level of nalidixic acid was raised from 0 to 20 mg dm^{-3}. At the highest concentration of nalidixic acid used, 60 mg dm^{-3}, the increase in DT was 15 h. However

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Test species	Concentration of nalidixic acid ($mg\ dm^{-3}$)	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
L.	0	9.1$\pm$0.9 #	966$\pm$ 42
monocytogenes	20	15.0$\pm$4.6	1185$\pm$165
NCTC 11994	40	19.4$\pm$0.7	931$\pm$138
	60	24.1$\pm$1.2	735$\pm$110
Lact.	0	7.0$\pm$0.7	751$\pm$103
lactis	20	7.8$\pm$0.6	734$\pm$ 81
NCIB 6681	40	9.8$\pm$1.2	668$\pm$162
	60	11.6$\pm$1.0 #	620$\pm$ 21
E. coli	0	7.3$\pm$0.6 #	658$\pm$ 74
NCIB 86	20	> 36	0
	40	> 36	0
	60	> 36	0
Ps.	0	7.5$\pm$0.3	579$\pm$115
fluorescens	20	7.7$\pm$0.3 #	525$\pm$ 93
NCIB 3756	40	8.6$\pm$1.0 #	531$\pm$ 84
	60	11.3$\pm$0.9 #	513$\pm$118
Mixed culture $	0	6.2$\pm$0.2	981$\pm$218
+ L.	20	7.2$\pm$0.2	1028$\pm$131
monocytogenes	40	7.2$\pm$0.2	1022$\pm$ 86
	60	10.4$\pm$1.0	705$\pm$ 47
Mixed culture $	0	6.2$\pm$0.5 #	989$\pm$173
- L.	20	6.8$\pm$0.5 #	1016$\pm$159
monocytogenes	40	7.3$\pm$0.3	887$\pm$131
	60	8.6$\pm$0.7	784$\pm$126

(N=6)

Key

$ = Each mixed culture consisted of:
Lact. lactis NCIB 6681,
E. coli NCIB 86 and
Ps. fluorescens NCIB 3756.
= One artefactual curve produced

Table 4.2 The effect of heat sterilised nalidixic acid on the impedimetric responses of individual test species and mixed cultures.

neither <u>Lact. lactis</u> nor <u>Ps. fluorescens</u> were affected to the same extent, the DT of both species being increased by less than 5 h at 60 mg dm^{-3} nalidixic acid. Only the electrical detection of <u>E. coli</u> was prevented during the time course of the assay by supplementing GNB with nalidixic acid.

No detectable difference was found between the electrical curves produced by the mixed culture in the presence and in the absence of <u>L. monocytogenes</u>. Therefore the sole impedimetric detection of this target organism was not possible using GNB supplemented with heat sterilised nalidixic acid. This was expected from extrapolation of the results obtained with individual organisms.

Electrical curves with a spike on the apex (see Fig. 3.10) were sometimes produced by <u>L. monocytogenes</u> and <u>Lact. lactis</u> in this work. However such curves were not observed in all experiments with pure cultures of these species or with mixed cultures containing these species. As "artefactual" electrical curves are easily distinguished from curves of normal morphology, they were not taken into consideration in the subsequent work reported. An investigation was undertaken to evaluate the effect of an alternative method of nalidixic acid sterilisation on this agent's influence on impedimetric responses. In these experiments nalidixic acid was filter sterilised, as described by Buchanan <u>et al</u>. (1987), prior to aseptic transfer to autoclaved GNB. Using routine

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conditions (see 2.3, 2.6 & 2.6.1) the impedimetric responses of the mixed cultures used in the previous experiment were monitored for 36 h in GNB supplemented with a range of concentrations of nalidixic acid. The results are tabulated in Table 4.3.

The alternative method of preparation of nalidixic acid resulted in a greater delay in DT for both mixed cultures at all concentrations of antimicrobial agent tested. This is evidence for the destruction of nalidixic acid during autoclaving, and this finding dictated the use of filter sterilising for all potentially heat labile selective agents in later experiments.

It can therefore be concluded that GNB supplemented with autoclaved or filter sterilised nalidixic acid does not allow the sole detection of <u>L. monocytogenes</u> from mixed cultures of microorganisms.

4.2.2 Investigation of thallous acetate as an agent selective for <u>L. monocytogenes</u>.

The selective agent thallous acetate (or thallium acetate) has previously been used for the isolation of Gram positive Lactic acid bacteria (Sharpe, 1952) as well as for <u>Listeria</u> spp. (Kramer & Jones, 1969).

Test species	Concentration of nalidixic acid ($mg\ dm^{-3}$)	Impedance DT (h) $\pm$ SD	Impedance magnitude (BUIC) $\pm$ SD
Mixed culture $ + L. monocytogenes	0	6.5$\pm$0.1	1123$\pm$130
	20	8.1$\pm$0.6	608$\pm$12
	40	10.4$\pm$1.6	704$\pm$52
	60	21.4$\pm$1.6	564$\pm$36
Mixed culture $ - L. monocytogenes	0	6.9$\pm$0.5	939$\pm$208
	20	7.9$\pm$0.4	813$\pm$85
	40	12.1$\pm$1.5	806$\pm$52
	60	17.2$\pm$4.9	705$\pm$120

(N=4)

Key

$ = Each mixed culture consisted of:
Lact. lactis NCIB 6681,
E. coli NCIB 86 and
Ps. fluorescens NCIB 3756.

Table 4.3 The effect of filter sterilised nalidixic acid on the impedimetric responses of mixed cultures of test species.

Using routine conditions (see 2.3 & 2.5) the growth of four test species was monitored by spectrophotometric assay in GNB supplemented with 0 to 3 g dm^{-3} of heat sterilised thallous acetate. This range of concentrations was chosen as 2 g dm^{-3} has been found to be effective in traditional methodology for the isolation of Listeria spp. (Kramer & Jones, 1969). Extreme care was taken with use of this poison (Gilman et al., 1985). The results of this work are given in Table 4.4.

Test species	Concentration of thallous acetate ($g \, dm^{-3}$)	Absorbance at 680 nm after 24 h
L.	0	0.32
monocytogenes	1	0.28
NCTC 11994	2	0.26
	3	0.22
Lact.	0	0.54
lactis	1	0.80 P
NCIB 6681	2	0.85 P
	3	0.70 P
E. coli	0	0.17
NCIB 86	1	0.00
	2	0.00
	3	0.00
Ps.	0	0.48
fluorescens	1	0.00
NCIB 3756	2	0.00
	3	0.00

(N=1)

Key

P = Thallous acetate precipitate disturbed

Table 4.4 Spectrophotometric assay of the effect of thallous acetate on the growth of test species in GNB.

All concentrations of thallous acetate tested were found to be effective against both Gram negative test organisms, Ps. fluorescens and E. coli. However, the highest concentration tested, 3 g dm^{-3}, was not effective against either of the Gram positive test organisms, L. monocytogenes and Lact. lactis. Thallous acetate may therefore prove to be a useful anti Gram negative agent for inclusion in a selective impedimetric medium.

A precipitate of thallous acetate was produced even

at 1 g dm^{-3}, the lowest concentration tested. As this deposit was accidentally disturbed in the tubes containing Lact. lactis no accurate absorbance readings could be obtained for this organism, although microbial growth was seen to be present at all concentrations.

The absorbance of the unsupplemented GNB controls of the four test species in Table 4.4 varied from those in Table 4.1. This variation may be due to carry over of differing amounts of stock culture into GNB for routine growth of the organisms prior to the selective toxic assay (see 2.3). Alternatively, it could be due to the use of different wavelengths in each case.

Further work was carried out to determine the effect of thallous acetate on the impedance curves produced by the four test species. Using routine conditions (see 2.3, 2.6 & 2.6.1) the impedimetric responses of the test species individually and in mixed culture were monitored for 36 h in GNB supplemented with 0 to 3 g dm^{-3} of thallous acetate. The results are tabulated in Table 4.5.

The lowest concentration of thallous acetate tested, 1 g dm^{-3}, inhibited the impedimetric responses of both Gram negative test species, E. coli and Ps. fluorescens, during the time course of the assay. The Gram positive organisms L. monocytogenes and Lact. lactis produced impedance curves in the supplemented medium, with those of the former species being of greater magnitude. However, the selective agent delayed the DT of both organisms to

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Test species	Concentration of thallous acetate ($g\ dm^{-3}$)	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
L. monocytogenes NCTC 11994	0	9.2$\pm$0.1	1018$\pm$ 87
	1	11.3$\pm$0.4	1032$\pm$144
	2	12.1$\pm$0.5	924$\pm$ 83
	3	11.7$\pm$0.5	1029$\pm$ 109
Lact. lactis NCIB 6681	0	7.1$\pm$0.4 #	726$\pm$132
	1	10.3$\pm$0.7	718$\pm$117
	2	10.5$\pm$0.4	705$\pm$ 83
	3	10.5$\pm$0.8 #	667$\pm$139
E. coli NCIB 86	0	8.1$\pm$0.7	686$\pm$6
	1	> 36	0
	2	> 36	0
	3	> 36	0
Ps. fluorescens NCIB 3756	0	7.0$\pm$0.8	845$\pm$185
	1	> 36	0
	2	> 36	0
	3	> 36	0
L. monocytogenes NCTC 11994 & Lact. lactis NCIB 6681	0	6.7$\pm$0.3	818$\pm$ 2
	1	10.1$\pm$0.5	823$\pm$ 22
	2	10.5$\pm$0.5	890$\pm$ 49
	3	9.7$\pm$0.2	781$\pm$ 19
Mixed culture $ + L. monocytogenes	0	6.6$\pm$0.1	912$\pm$ 32
	1	10.5$\pm$0.2	572$\pm$ 40
	2	10.0$\pm$0.4	652$\pm$ 5
	3	11.2$\pm$0.1	672$\pm$ 2
Mixed culture $ - L. monocytogenes	0	6.6$\pm$0.1	844$\pm$ 18
	1	10.3$\pm$0.3	485$\pm$ 6
	2	10.9$\pm$0.1	508$\pm$ 19
	3	12.2$\pm$0.6	470$\pm$ 33

(N=4)

Key

$ = Each mixed culture consisted of:
Lact. lactis NCIB 6681,
E. coli NCIB 86 and
Ps. fluorescens NCIB 3756.
= One artefactual curve produced.

Table 4.5 The effect of thallous acetate on the impedimetric responses of individual test species and mixed cultures.

approximately the same extent at all concentrations tested. This finding together with the observation of a white precipitate at all concentrations suggests that the GNB solution may be saturated with thallous acetate even at 1 g dm^{-3}. However, from the absorbance of L. monocytogenes (Table 4.4) it was seen that an increase in thallous acetate from 1 to 3 g dm^{-3} caused a corresponding but small decrease in the growth of this test species indicating the thallous acetate was not saturated at these concentrations despite the precipitation.

The difference between the optimal concentration of thallous acetate determined by Kramer and Jones (1969) and that observed in this work can be credited to variation in the test species employed and to the method of analysis used. Additionally the aim of this work was to find a concentration of thallous acetate which prevented non Listeria test species from reaching the threshold level required to produce electrical curves within 36 h and not to prevent the formation of colonies by those species.

The high impedance magnitude of L. monocytogenes in pure culture was not observed in the mixed culture containing L. monocytogenes (Table 4.5). This suggests that the presence of mixed cultures of organisms can mediate the characteristics of the impedance curves thought to be attributable to target organisms within those mixed cultures.

An investigation was carried out to determine whether lower concentrations of thallous acetate than those previously used (Table 4.5) were as effective against Gram negative organisms. Using the routine conditions (see 2.3, 2.6 & 2.6.1) the impedimetric responses of Ps. fluorescens and L. monocytogenes were monitored for 36 h in GNB supplemented with 0 to 0.6 g dm^{-3} thallous acetate. The results are given in Table 4.6.

The lowest concentration of thallous acetate which inhibited the impedimetric detection of Gram negative Ps. fluorescens was 0.06 g dm^{-3}. This concentration had a negligible effect on the DT of L. monocytogenes, causing less than a 1.0 h increase. However, the effectiveness of this concentration of thallous acetate in preventing one particular Gram negative test organism from producing a detection curve may not be representative of Gram negative organisms generally.

It was therefore concluded that thallous acetate could possibly be used to eliminate the detection curves of Gram negative organisms without causing an excessive delay in the DT of L. monocytogenes. In addition, as very few artefactual curves were produced in thallous acetate supplemented GNB this agent may have a curve stabilising effect. However, because of its mammalian toxicity it would not be used in preference to an alternative agent with similar characteristics.

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| Test species | Concentration of thallous acetate (g dm^{-3}) | Impedance | |
		DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
L. monocytogenes NCTC 11994	0	9.5$\pm$0.5	1097$\pm$ 13
	0.02	9.8$\pm$0.1	1202$\pm$ 26
	0.04	10.6$\pm$0.3	1162$\pm$ 15
	0.06	10.3$\pm$0.1	1224$\pm$ 13
	0.20	15.4$\pm$1.7	1096$\pm$ 49
	0.40	17.2$\pm$0.8	1040$\pm$ 10
	0.60	18.5 #	698
Ps. fluorescens NCIB 3756	0	7.6$\pm$0.4	655$\pm$ 35
	0.02	13.8$\pm$0.2	543$\pm$ 7
	0.04	24.5$\pm$0.6	431$\pm$ 3
	0.06	> 36	0
	0.20	> 36	0
	0.40	> 36	0
	0.60	> 36	0

(N=2)

Key
= One artefactual curve produced

Table 4.6 The effect of thallous acetate on the impedimetric responses of **L. monocytogenes** and **Ps. fluorescens**.

4.2.3 Investigation of moxalactam as an agent selective for **L. monocytogenes**

A number of cephalosporins, such as ceftazidime, cefotetan and moxalactam, have been used for the selective isolation of **Listeria** (Curtis **et al.**, 1989b). The third generation cephalosporins, which include moxalactam, have been reported to have a broad spectrum of activity although they are less active against Gram positive organisms (Gilman **et al.**, 1985).

Using the routine conditions (see 2.3 & 2.5) the
growth of four test species was monitored by
spectrophotometric assay in GNB supplemented with 0 to
60 mg dm^{-3} moxalactam. The results are shown in Table 4.7.

The growth of the Gram negative organisms
Ps. fluorescens and E. coli was completely inhibited at
20 mg dm^{-3}, the lowest concentration of moxalactam tested.
The growth of both Gram positive organisms,
L. monocytogenes and Lact. lactis, was reduced at all
concentrations of moxalactam used, with the latter
organism showing greater sensitivity.

Test species	Concentration of moxalactam (mg dm^{-3})	Absorbance at 680 nm after 24 h
L. monocytogenes NCTC 11994	0	0.50
	20	0.44
	40	0.38
	60	0.16
Lact. lactis NCIB 6681	0	1.20
	20	0.80
	40	0.70
	60	0.18
E. coli NCIB 86	0	0.85
	20	0.00
	40	0.00
	60	0.00
Ps. fluorescens NCIB 3756	0	0.80
	20	0.03
	40	0.03
	60	0.05

(N=1)

Table 4.7 Spectrophotometric assay of the effect of
moxalactam on the growth of test species in GNB.

Further work was carried out to determine the effect
of moxalactam on the impedance curves of the test
organisms. Using the routine conditions (see 2.3, 2.6 &
2.6.1) the impedimetric responses of the four test species
individually and in mixed culture were monitored for 36 h
in GNB supplemented with 0 to 60 mg dm^{-3} of moxalactam.
The results are recorded in Table 4.8.

The lowest concentration of moxalactam used,
20 mg dm^{-3}, inhibited the impedimetric detection of the
three non Listeria test species examined both individually
and in mixed culture. One false positive detection curve
was produced by the mixed culture without L. monocytogenes
at 20 mg dm^{-3}; however the DT in this reaction well was
delayed extensively. Such a false detection could be
easily prevented by a small increase in the concentration
of moxalactam. This should not delay the detection of
L. monocytogenes unduly as none of the concentrations of
moxalactam used in this experiment caused more than a
two h increase in its DT. The target organism itself was
consistently detected in both pure and mixed culture.
Furthermore it produced impedance curves of good
morphology and reasonable magnitude at all concentrations
of moxalactam tested.

The medium formulation used in this experiment
produced artefactual curves in 8 out of 128 reaction wells
(see Fig. 3.10 for examples). However, these curves
occurred in both supplemented and unsupplemented GNB

Test species (& number of samples)	Concentration of moxalactam (mg dm^{-3})	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
L. **monocytogenes** NCTC 11994 (N=4)	0	9.5$\pm$0.5	1079$\pm$126
	20	10.0$\pm$0.3 #	1242$\pm$ 73
	40	11.1$\pm$1.0	1068$\pm$195
	60	10.7$\pm$0.1 *	911$\pm$ 24
Lact. **lactis** NCTC 6681 (N=4)	0	9.0$\pm$0.8 #	1277$\pm$740
	20	> 36	0
	40	> 36	0
	60	> 36	0
E. coli NCIB 86 (N=4)	0	8.5$\pm$0.1 *	936$\pm$ 79
	20	> 36	0
	40	> 36	0
	60	> 36	0
Ps. **fluorescens** NCIB 3756 (N=4)	0	7.6$\pm$0.6	857$\pm$ 36
	20	> 36	0
	40	> 36	0
	60	> 36	0
Mixed culture § + **L.** **monocytogenes** (N=8)	0	6.3$\pm$0.3	1159$\pm$176
	20	9.8$\pm$0.6	1369$\pm$419
	40	11.6$\pm$1.2 #	1112$\pm$113
	60	13.9$\pm$1.7 #	955$\pm$195
Mixed culture § - **L.** **monocytogenes** (N=8)	0	7.8$\pm$1.0	1162$\pm$204
	20	26.4 (1) ◊	822
		> 36 (7)	0
	40	> 36	0
	60	> 36	0

<u>Key</u>

\# = One artefactual curve produced
* = Two artefactual curves produced
◊ = Number of curves of each type in brackets
§ = Each mixed culture consisted of:
   <u>Lact. lactis</u> NCIB 6681, <u>E. coli</u> NCIB 86
   and <u>Ps. fluorescens</u> NCIB 3756.

Table 4.8 The effect of moxalactam on the impedimetric responses of individual test species and mixed cultures.

indicating that moxalactam was not the causative agent.

A number of problems were encountered when this set of experiments was repeated with a batch of moxalactam which was approximately 5 months old (I Hancock, unpublished data). This indicates that the agent is unstable and loses activity on long term storage.

As moxalactam had been found to be effective against three non <u>Listeria</u> species the range of test microorganisms was then increased to include five new species of lactic acid bacteria. These species are commonly found in dairy products susceptible to contamination by <u>Listeria</u> spp. Higher concentrations of the selective agent were used with the aim of achieving the sole impedimetric detection of <u>L. monocytogenes</u>.

Using routine conditions (see 2.3 & 2.5) the growth of eight test species was measured by spectrophotometric assay in GNB supplemented with 0 to 120 mg dm^{-3} moxalactam. The results are tabulated in Table 4.9.

It was found that the lowest concentration of moxalactam tested, 40 mg dm^{-3}, prevented the growth of the Gram negative microorganisms, <u>E. coli</u> and <u>Ps. fluorescens</u>, and two of the Gram positive microorganisms, <u>Lact. lactis</u> subsp <u>cremoris</u> and <u>Leuc. mesenteroides</u> subsp <u>cremoris</u>. However, at the highest concentration used, 120 mg dm^{-3}, moxalactam inhibited the growth of <u>L. monocytogenes</u> to a greater extent than that of the remaining three lactic acid bacteria, <u>Strep. agalactiae</u>, <u>Ent. faecalis</u> and <u>Strep. salivarius</u>.

Test species	Concentration of moxalactam ($mg\ dm^{-3}$)	Absorbance at 680 nm after 24 h
L. monocytogenes NCTC 11994	0	0.38
	40	0.15
	80	0.20
	120	0.09
Strep. agalactiae NCIB 8778	0	0.63
	40	0.58
	80	0.45
	120	0.29
Lact. lactis subsp cremoris NCIB 8662	0	0.56
	40	0.00
	80	0.00
	120	0.00
Ent. faecalis NCIB 775	0	0.75
	40	0.64
	80	0.30
	120	0.13
Strep. salivarius NCIB 8883	0	0.60
	40	0.59
	80	0.45
	120	0.30
Leuc. mesenteroides subsp cremoris NCIB 12008	0	0.41
	40	0.00
	80	0.00
	120	0.00
E. coli NCIB 86	0	0.50
	40	0.00
	80	0.00
	120	0.00
Ps. fluorescens NCIB 3756	0	0.71
	40	0.01
	80	0.00
	120	0.00

(N=1)

Table 4.9 Spectrophotometric assay of the effect of moxalactam on the growth of test species in GNB.

In the experiments so far reported the diammonium salt of moxalactam had been evaluated for its potential as an agent selective for L. monocytogenes. At this stage in the research its commercial production was discontinued so, on the advice of Sigma's Technical Service Department, the sodium salt was used instead in all subsequent experiments. As the latter type of moxalactam differed both chemically and in physical appearance from the original type, its influence on the electrical curves of the standard four test species was therefore reexamined.

Using routine conditions (see 2.3, 2.6 & 2.6.1) the impedimetric responses of the four test species were monitored in GNB supplemented with 0 to 100 mg dm^{-3} of the sodium salt of moxalactam. The results are tabulated in Table 4.10.

The effect of the sodium salt of moxalactam on the impedimetric responses of the test species was found to be similar to that of the diammonium salt used in previous work (Table 4.8). The new type of moxalactam inhibited the impedimetric detection of all three non Listeria species although Lact. lactis produced one late detection curve at 20 mg dm^{-3}. This could have been due to a heavy inoculum of stock culture being taken for overnight incubation in GNB prior to use on this occasion. Such late detection curves were prevented by the use of a higher concentration of moxalactam.

Test species	Concentration of moxalactam ($mg\ dm^{-3}$)	Impedance DT (h) ± SD	Impedance magnitude (BUIC) ± SD
L.	0	9.7±0.2	1135± 72
monocytogenes	20	9.9±0.3	1191± 74
NCTC 11994	40	10.5±0.9	1127± 41
	60	11.4±0.4	1089± 30
	80	13.1±0.1	1036± 2
	100	16.2±0.1	862± 94
Lact.	0	9.8±0.1	730± 11
lactis	20	34.6(1) ⑤	93
NCIB 6681		> 36 (2),#	0
	40	> 36	0
	60	> 36	0
E. coli	0	5.1±0.0	771± 25
NCIB 86	20	> 36	0
	40	> 36	0
	60	> 36	0
Ps.	0	9.3±1.3	481±117
fluorescens	20	> 36	0
NCIB 3756	40	> 36	0
	60	> 36	0

(N=4)

Key
\# = One artefactual curve produced.
⑤ = Number of curves of each type in brackets.

Table 4.10 The effect of the sodium salt of moxalactam on the impedimetric responses of four test species.

It was concluded that moxalactam could be useful for incorporation into a selective impedimetric medium as it is active against both Gram negative and a number of Gram positive test species (Table 4.9). It does not hinder the quality of impedance curves or increase the DT of the target organism L. monocytogenes unduly (Tables 4.8 & 4.10).

4.2.4 Investigation of lithium chloride as an agent selective for L. monocytogenes

The toxic effects of lithium chloride are related to the imperfect substitution of lithium for other cations such as sodium and potassium ions which normally generate electrochemical gradients and maintain osmotic balance (Oehme, 1978).

Using routine conditions (see 2.3 & 2.5) the growth of seven test species was monitored by spectrophotometric assay in GNB supplemented with 0 to 6 g dm^{-3} lithium chloride. This range of concentrations was chosen for evaluation as Lee & McClain (1986) used 5 g dm^{-3} lithium chloride in their selective plating medium. The results are recorded in Table 4.11.

Lithium chloride was not found to be an effective selective agent at the concentrations tested as it allowed the growth of all test microorganisms examined except Lact. lactis.

In conjunction with the spectrophotometric assays, work was carried out to evaluate the effect of lithium chloride on the impedimetric responses of test species. Using routine methods (see 2.3, 2.6 & 2.6.1) eight test microorganisms were monitored in GNB supplemented with 0 to 6 g dm^{-3} lithium chloride. The results are tabulated in Table 4.12.

Test species	Concentration of lithium chloride (g dm^{-3})	Absorbance at 680 nm after 24 h
L. monocytogenes NCTC 11994	0	0.28
	2	0.25
	4	0.23
	6	0.22
Lact. lactis NCIB 6681	0	0.89
	2	0.80
	4	0.20
	6	0.04
Strep. agalactiae NCIB 8778	0	0.81
	2	0.70
	4	0.65
	6	0.58
Ent. faecalis NCIB 775	0	0.83
	2	0.76
	4	0.70
	6	0.68
Strep. salivarius NCIB 8883	0	0.82
	2	0.72
	4	0.49
	6	0.30
E. coli NCIB 86	0	0.32
	2	0.24
	4	0.35
	6	0.34
Ps. fluorescens NCIB 3756	0	0.40
	2	0.38
	4	0.40
	6	0.38

(N=1)

Table 4.11 Spectrophotometric assay of the effect of lithium chloride on the growth of test species in GNB.

Test species	Concentration of lithium chloride (g dm^{-3})	DT (h) ± SD	Impedance magnitude (BUIC) ± SD
L.	0	10.4±3.0	961± 52
monocytogenes	2	10.5 #	1357
NCTC 11994	4	10.0±0.2	1513± 45
	6	9.7±0.2	1503± 11
Lact.	0	9.8±0.0	1098± 21
lactis	2	9.5±0.3	1209± 6
NCIB 6681	4	8.0±2.0	1370± 19
	6	8.8 #	1578
Ent.	0	7.6±0.1	1093± 69
faecalis	2	7.7±0.2	1320± 28
NCIB 775	4	7.1 #	1621
	6	8.3 #	1675
Strep.	0	6.2±0.1	1183± 9
agalactiae	2	6.5±0.3	1399± 55
NCIB 8778	4	6.3±0.0	1608± 96
	6	6.7 #	1649
Strep.	0	6.1±0.0	1104± 1
salivarius	2	6.3±0.2	1439± 98
NCIB 8883	4	6.2 #	1541
	6	6.5±0.2	1594± 624
Lactob.	0	33.1±1.6	217±132
casei	2	> 36	0
subsp rhamnosus	4	> 36	0
NCIB 6375	6	> 36	0
E. coli	0	7.5±0.2	716± 41
NCIB 86	2	8.3±0.3	881± 80
	4	*	
	6	14.8 #	928
Ps.	0	7.1±0.2	698± 8
fluorescens	2	7.1 #	798
NCIB 3756	4	7.2 #	818
	6	7.5±0.0	917± 50

(N=2)

Key
= One artefactual curve produced
* = Two artefactual curves produced

Table 4.12 The effect of lithium chloride on the impedimetric responses of test species.

The DT's of all test microorganisms except
Lactob. casei subsp rhamnosus and E. coli were unaffected
even at the highest concentration of lithium chloride
tested, thus indicating a far greater concentration was
required for the selective inhibition of most test
species. As Lactob. casei subsp rhamnosus produced a late
DT in unsupplemented GNB and E. coli produced a number of
artefactual curves in the higher concentrations of
supplemented GNB, it is difficult to determine the exact
effects of the lithium chloride on these test species. It
must be noted that while the growth of Lact. lactis was
affected by lithium chloride (Table 4.11) the impedimetric
responses of this species were not inhibited at equivalent
concentrations of the selective agent (Table 4.12). This
indicates that either there were discrepancies between the
inoculum concentrations in the two assays or that the
spectrophotometric assay is not a good predictor of the
impedimetric responses of this organism.

It was observed that the addition of lithium chloride
to GNB improved the magnitude of the impedance curves,
thus making the interpretation of the curves easier (see
2.6.1). The potential of this chemical as a curve
improving agent may be limited, however, as a large number
of artefactual curves were produced particularly in the
higher concentrations of lithium chloride supplemented
GNB.

Further work was then carried out to determine
whether lower concentrations of lithium chloride would

improve the quality of impedance curves. Using routine
conditions (see 2.3, 2.6 & 2.6.1) the impedimetric
responses of two test species were monitored for 36 h in
GNB supplemented with 0 to 1.0 g dm^{-3} lithium chloride.
The results are tabulated (Table 4.13).

Lithium chloride concentrations in the range 0.2 to
1.0 g dm^{-3} were found to improve the magnitude and, to a
lesser extent, the morphology of the impedance curves
produced by L. monocytogenes and Ps. fluorescens. A much
lower proportion of artefactual curves were observed in
this set of experiments than had been previously found
when higher concentrations of lithium chloride were used
(Table 4.12).

It was concluded that while lithium chloride did not
allow the selection of L. monocytogenes from mixed
culture, low concentrations of this agent might be used in
a selective impedimetric medium to improve the quality of
the curves produced by the target organism.

4.2.5 Investigation of phenylethanol as an agent selective
for L. monocytogenes

Phenylethanol has been used as a selective agent in
traditional plate methods of isolating L. monocytogenes.
It has been found to suppress the growth of some
Pseudomonas spp. (McBride & Girard, 1960; Mossel et al.,
1977).

```
/-----------------------------------------------------------\
|  Test        Concentration          Impedance             |
|  species     of lithium       DT           magnitude      |
|              chloride         (h) + SD      (BUIC) + SD    |
|              (g dm^-3)                                     |
|-----------------------------------------------------------|
|  L.          0.0             8.6+ 0        1086+ 27        |
|  monocytogenes  0.2          8.4+0.1       1590+ 76        |
|  NCTC 11994  0.4             8.4 #         2107            |
|              0.6             8.0+0.1       2396+ 11        |
|              1.0             9.2+0.3       1234+ 20        |
|-----------------------------------------------------------|
|  Ps.         0.0             7.2+0.1        709+  8        |
|  fluorescens 0.2             7.8+0.1       1033+ 10        |
|  NCIB 3756   0.4             7.7+0.3       1059+ 56        |
|              0.6             8.4+0.5       1147+  7        |
|              1.0             7.8+1.2        568+ 11        |
\-----------------------------------------------------------/
                                          (N=2)
                                   Key
                 # = One artefactual curve produced
```

Table 4.13 The effect of lithium chloride on the
impedimetric responses of two test species.

A spectrophotometric assay was attempted to evaluate
the antimicrobial spectrum of phenylethanol. However,
although previous workers had used similar concentrations
(Kramer & Jones, 1969), phenylethanol was found in this
work to be immiscible in GNB at 2 to 6 g dm^{-3}. Use of a
small amount of inorganic solvent to promote dispersion of
phenylethanol was deliberated, but evaluating potential
additive or synergistic effects was not thought desirable
at this stage as other agents, such as thallous acetate
and moxalactam, had already been found to be capable of
eliminating electrical signals produced by Gram negative
Pseudomonas spp. Furthermore, Adak et al. (1987b) showed
even minute quantities of solvents affected electrical
signals. It was therefore decided to discontinue the
investigation of phenylethanol at this point.

4.2.6 Investigation of potassium tellurite as an agent selective for L. monocytogenes

Gray et al. (1950) reported that potassium tellurite at concentrations of 0.1 to 1.0 g dm^{-3} was effective against Gram negative organisms. Leighton (1979) advised the use of 0.5 g dm^{-3} for the same purpose.

Using the routine conditions (see 2.3 & 2.5) the growth of four test species was determined spectrophotometrically in GNB supplemented with 0 to 1.25 g dm^{-3} potassium tellurite. The results are tabulated in Table 4.14.

The Gram negative species Ps. fluorescens and E. coli were both inhibited at the lowest concentration of potassium tellurite tested, 0.25 g dm^{-3}. At the highest concentration of potassium tellurite, 1.25 g dm^{-3}, the growth of L. monocytogenes was inhibited less than that of the other Gram positive organism, Lact. lactis. Both L. monocytogenes and Lact. lactis were observed to produce a black colour change in the potassium tellurite supplemented GNB.

Further work was undertaken to ascertain the effect of lower concentrations of potassium tellurite on impedance detection. Using the routine conditions (see 2.3, 2.6 & 2.6.1) the impedimetric responses of L. monocytogenes and Ps. fluorescens were monitored for 36 h in GNB supplemented with 0 to 0.6 g dm^{-3} potassium tellurite. The results are tabulated (Table 4.15).

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Test species	Concentration of potassium tellurite $(g\ dm^{-3})$	Absorbance at 680 nm after 24 h
L.	0	0.44
monocytogenes	0.25	0.64 æ
NCTC 11994	0.50	0.91 æ
	0.75	0.29 æ
	1.00	0.32 æ
	1.25	0.20 æ
Lact.	0	0.16
lactis	0.25	0.32 æ
NCIB 6681	0.50	0.26 æ
	0.75	0.25 æ
	1.00	0.16 æ
	1.25	0.04 æ
E. coli	0	0.27
NCIB 86	0.25	0.01
	0.50	0.00
	0.75	0.00
	1.00	0.00
	1.25	0.00
Ps.	0	0.42
fluorescens	0.25	0.00
NCIB 3756	0.50	0.00
	0.75	0.00
	1.00	0.00
	1.25	0.00

(N=1)

Key

æ = Colour change to black.

Table 4.14 Spectrophotometric assay of the effect of potassium tellurite on the growth of test species in GNB.

Potassium tellurite inhibited <u>Ps. fluorescens</u> at the
lowest concentration tested, 0.02 g dm^{-3}, whilst
<u>L. monocytogenes</u> had an approximate 1 h delay in DT at
0.02 g dm^{-3} and was resistant up to 0.04 g dm^{-3}.
Unfortunately the presence of potassium tellurite in GNB
greatly reduced impedance magnitude. This made the
interpretation of the curves very difficult and
effectively precluded the use of potassium tellurite in a
selective impedimetric assay for <u>L. monocytogenes</u>.

Test species	Concentration of potassium tellurite (g dm^{-3})	DT (h) ± SD	Impedance magnitude (BUIC) ± SD
<u>L.</u>	0	9.4±0.0	907± 19
<u>monocytogenes</u>	0.02	10.8±0.2	201± 2
NCTC 11994	0.04	14.7±0.1	74± 1
	0.06	> 36	0
	0.20	> 36	0
	0.40	> 36	0
	0.60	> 36	0
<u>Ps.</u>	0	7.1±0.1	669± 5
<u>fluorescens</u>	0.02	> 36	0
NCIB 3756	0.04	> 36	0
	0.06	> 36	0
	0.20	> 36	0
	0.40	> 36	0
	0.60	> 36	0

(N=2)

Table 4.15 The effect of potassium tellurite on the
impedimetric responses of the test species
<u>L. monocytogenes</u> and <u>Ps. fluorescens</u>.

4.2.7 Investigation of oxolinic acid as an agent selective for L. monocytogenes.

Oxolinic acid, a quinolone, is a congener of nalidixic acid. It is similar to nalidixic acid in its mechanism of action and spectrum of antimicrobial activity, although it is supposedly two to four times more active than nalidixic acid in vitro (Gilman et al., 1985).

Oxolinic acid was found to produce a fine precipitate in aqueous solution. As filter sterilisation would affect the final concentration in GNB, this agent was sterilised by autoclaving. This resulted in an aqueous solution of unknown concentration, although the total content of the suspension of oxolinic acid was at the concentration stated.

As the particulate nature of oxolinic acid would influence the absorbance readings obtained in the spectrophotometric assay (see methods 2.5), this assay was not performed. However the effect of oxolinic acid on the electrical responses of test species was investigated. Using the routine conditions (see 2.3, 2.6 & 2.6.1) the impedimetric responses of eight test species were monitored for 36 h in GNB supplemented with 0 to 20 mg dm^{-3} oxolinic acid. These concentrations were chosen as 60 mg dm^{-3} nalidixic acid had delayed the DT of L. monocytogenes by 15 h (Table 4.2). The results are tabulated (Table 4.16).

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Test species	Concentration of oxolinic acid ($mg\ dm^{-3}$)	Impedance	
		DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
L. monocytogenes NCTC 11994	0	9.3 ± 0.2	1168 ± 200
	6	9.0 ± 0.1	925 ± 1
	12	9.5 ± 1.0	905 ± 3
	20	9.5 ± 0.3	1000 ± 73
Lact. lactis NCIB 6681	0	8.4 ± 0.2	815 ± 5
	6	8.1 ± 0.1	802 ± 2
	12	9.2 ± 0.2	822 ± 17
	20	9.3 ± 0.5	852 ± 6
Ent. faecalis NCIB 775	0	7.0 ± 0.1	882 ± 17
	6	7.2 #	1021
	12	9.6 ± 0.9	904 ± 12
	20	8.6 ± 0.3	913 ± 6
Strep. salivarius NCIB 8883	0	6.3 ± 0.0	785 ± 52
	6	6.5 ± 0.0	848 ± 20
	12	9.1 ± 0.2	739 ± 46
	20	10.5 ± 0.0	763 ± 47
B. cereus NCIB 8122	0	7.0 ± 0.0	644 ± 22
	6	> 36	0
	12	> 36	0
	20	> 36	0
B. subtilis NCIB 8703	0	10.3 #	915
	6	> 36 #	0
	12	> 36	0
	20	> 36	0
E. coli NCIB 86	0	7.3 ± 0.2	1194 ± 278
	6	11.7 #	133
	12	17.0 ± 0.9	1067 ± 89
	20	> 36	0
Ps. fluorescens NCIB 3756	0	9.0 ± 0.4	880 ± 267
	6	9.1 ± 0.1	773 ± 3
	12	24.2 #	605
	20	*	

(N=2)

Key
= One artefactual curve produced
* = Two artefactual curves produced

Table 4.16 The effect of oxolinic acid on the impedimetric responses of test species in GNB.

Both <u>L. monocytogenes</u> and the other lactic acid bacteria produced impedimetric signals at all concentrations of oxolinic acid tested. However even at 20 mg dm^{-3} the oxolinic acid supplemented GNB yielded poor quality impedance curves with a rounded acceleration (see Fig. 3.3). As the shoulder of these curves was indistinct, curve interpretation was very difficult. The impedimetric responses of the <u>Bacillus</u> spp. were eliminated at the lowest concentration of oxolinic acid examined, 6 mg dm^{-3}, and those of <u>E. coli</u> were inhibited at 20 mg dm^{-3}. Due to the production of artefactual curves it is not certain that the latter concentration of oxolinic acid was also inhibitory to <u>Ps. fluorescens</u>, however as a late detection curve was produced at 12 mg dm^{-3} this is likely to be the case. An unexpected observation was the difference in relative sensitivity of <u>L. monocytogenes</u> and <u>Ps. fluorescens</u> to nalidixic acid (Table 4.2) and to oxolinic acid (Table 4.16).

Further work was carried out to assess the effect of lower concentrations of oxolinic acid on the impedimetric responses of a range of test species. These experiments were performed to determine whether concentrations capable of eliminating the electrical signals produced by <u>Bacillus</u> spp. affected the quality of impedance curves produced by <u>L. monocytogenes</u>. Using the routine conditions (see 2.3, 2.6 & 2.6.1) the impedimetric responses of seven test species were monitored for 36 h in GNB supplemented with 0 to 6 mg dm^{-3} oxolinic acid. The results are tabulated (Table 4.17).

Test species	Concentration of oxolinic acid (mg dm^{-3})	Impedance DT (h) $\pm$ SD	magnitude (BUIC)_ SD
L. monocytogenes NCTC 11994	0	9.0$\pm$0.0	918$\pm$ 34
	2	10.1 #	987
	4	9.4$\pm$0.4	1066$\pm$ 43
	6	8.6$\pm$0.3	1037$\pm$103
Lact. lactis NCIB 6681	0	8.9$\pm$0.2	711$\pm$ 7
	2	9.4$\pm$0.0	661$\pm$ 9
	4	9.1$\pm$0.4	747$\pm$ 4
	6	8.7$\pm$0.4	695$\pm$ 68
Ent. faecalis NCIB 775	0	7.1 #	785
	2	7.6$\pm$1.0	884$\pm$ 32
	4	7.6$\pm$0.5	838$\pm$ 33
	6	8.2$\pm$0.6	697$\pm$ 61
B. cereus NCIB 8122	0	5.9$\pm$0.3	703$\pm$ 58
	2	*	
	4	15.8$\pm$0.8	615$\pm$106
	6	> 36	0
B. circulans NCIB 7578	0	8.3$\pm$0.0	553$\pm$ 66
	2	*	
	4	17.0$\pm$0.7	525$\pm$ 7
	6	> 36 #	0
B. subtilis NCIB 8903	0	7.4$\pm$0.1	723$\pm$181
	2	7.5 #	498
	4	14.5$\pm$0.3	525$\pm$ 28
	6	> 36	0
Ps. fluorescens NCIB 3756	0	8.0$\pm$1.2	722$\pm$153
	2	7.1$\pm$0.2	638$\pm$ 45
	4	8.7$\pm$0.5	593$\pm$ 97
	6	8.4 #	616

(N=2)

Key
= one artefactual curve produced
* = two artefactual curves produced

Table 4.17 The effect of oxolinic acid on the impedimetric responses of test species in GNB.

6 mg dm^{-3} oxolinic acid was found to be the lowest concentration that prevented the detection of the Bacillus spp. As expected the DT of L. monocytogenes, the lactic acid bacteria and Ps. fluorescens was not delayed at any of the concentrations of oxolinic acid tested.

In spite of its potentially useful activity against Bacillus spp., it was concluded that oxolinic acid would be unlikely to be included in a selective impedimetric medium for L. monocytogenes for two reasons: firstly because of its solubility problems and secondly because of the poor quality impedance curves produced by the target species in oxolinic acid supplemented GNB.

4.2.8 Investigation of acriflavine hydrochloride as an agent selective for L. monocytogenes.

Acriflavine hydrochloride has been used by many researchers in Listeria selective media, for example, Ralovich (1984) used 20 mg dm^{-3} and Farber et al. (1987), 15 mg dm^{-3}. Dominguez et al. (1984) initially used 18.7 mg dm^{-3}, but found this to inhibit the growth of L. grayi, L. murrayi and J. denitrificans, and, in order to isolate these species, they had to reduce the concentration of acriflavine to 12 mg dm^{-3}.

As this research is aimed at the rapid detection of pathogenic Listeria spp., especially L. monocytogenes, it would be useful to use concentrations of acriflavine hydrochloride which inhibit the non pathogenic species of

Listeria. It was estimated that these should lie in the range 20 to 60 mg dm^{-3}. Using the routine conditions (see 2.3 & 2.5) the growth of eight test species was therefore determined by spectrophotometric assay in GNB supplemented with 0 to 60 mg dm^{-3} acriflavine hydrochloride. The results are tabulated in Table 4.18.

As acriflavine hydrochloride is a fluorescein dye there was some difficulty in evaluating microbial growth by this assay. However 20 mg dm^{-3} was determined to be inhibitory to the growth of all the Gram positive test species except L. monocytogenes. This concentration was also found to be effective against Gram negative E. coli, but not Ps. fluorescens.

Further work was then carried out to determine the influence of acriflavine hydrochloride on the electrical responses of a number of test species. Using the routine procedures (see 2.3, 2.6 & 2.6.1) the impedimetric responses of nine test species were monitored for 36 h in GNB supplemented with 0 to 60 mg dm^{-3} acriflavine hydrochloride. The results are tabulated (Table 4.19).

Of the test microorganisms, only L. monocytogenes and Ps. fluorescens produced impedimetric responses at 20 mg dm^{-3} acriflavine hydrochloride. The responses of L. monocytogenes were inhibited at the highest concentration of agent tested, 60 mg dm^{-3}, whilst those of Ps. fluorescens were resistant to all concentrations tested.

Test species	Concentration of acriflavine hydrochloride ($mg\ dm^{-3}$)	Absorbance at 680 nm after 24 h
L.	0	0.33
monocytogenes	20	0.05
NCTC 11994	40	0.01
	60	0.00
Lact.	0	0.79
lactis	20	0.00
NCIB 6681	40	0.00
	60	0.00
Strep.	0	0.63
agalactiae	20	0.00
NCIB 8778	40	0.00
	60	0.00
Lact. lactis	0	0.36
subsp cremoris	20	0.00
NCIB 8662	40	0.00
	60	0.00
Ent.	0	0.69
faecalis	20	0.00
NCIB 775	40	0.00
	60	0.00
Leuc.	0	0.61
mesenteroides	20	0.00
subsp cremoris	40	0.00
NCIB 12008	60	0.00
E. coli	0	0.48
NCIB 86	20	0.00
	40	0.00
	60	0.00
Ps.	0	0.62
fluorescens	20	0.16
NCIB 3756	40	0.00
	60	0.00

(N=1)

Table 4.18 Spectrophotometric assay of the effect of acriflavine hydrochloride on the growth of test species in GNB.

Test species	Concentration of acriflavine hydrochloride ($mg\ dm^{-3}$)	Impedance DT (h) $\pm$ SD	Impedance magnitude (BUIC) $\pm$ SD
L. monocytogenes NCTC 11994	0	10.4$\pm$0.5	956$\pm$160
	20	10.8$\pm$0.7	1025$\pm$247
	40	21.4$\pm$8.9	759$\pm$ 22
	60	> 36	0
Lact. lactis NCIB 6681	0	10.1$\pm$1.0 #	880$\pm$136
	20	> 36	0
	40	> 36	0
	60	> 36	0
Ent. faecalis NCIB 775	0	6.6$\pm$0.1	937$\pm$92
	20	> 36	0
	40	> 36	0
	60	> 36	0
Strep. salivarius NCIB 8883	0	6.3$\pm$0.1	957$\pm$113
	20	> 36	0
	40	> 36	0
	60	> 36	0
Strep. agalactiae NCIB 8778	0	7.0$\pm$0.7	823$\pm$ 77
	20	> 36	0
	40	> 36	0
	60	> 36	0
Lact. lactis subsp cremoris NCIB 8662	0	10.4$\pm$1.0	789$\pm$ 93
	20	> 36	0
	40	> 36	0
	60	> 36	0
Leuc. mesenteroides subsp cremoris NCIB 12008	0	14.9$\pm$1.2	923$\pm$130
	20	> 36	0
	40	> 36	0
	60	> 36	0
E. coli NCIB 86	0	7.6$\pm$0.1	955$\pm$ 20
	20	> 36	0
	40	> 36	0
	60	> 36	0
Ps. fluorescens NCIB 3756	0	6.2$\pm$1.4	769$\pm$ 9
	20	7.4$\pm$0.3	781$\pm$146
	40	13.1$\pm$3.0	621$\pm$ 15
	60	30.5$\pm$2.0 *	228$\pm$114

Key   (N=4)
= one artefactual curve produced
* = two artefactual curves produced

Table 4.19 The effect of acriflavine hydrochloride on the impedimetric responses of test species in GNB.

Increasing the concentration of acriflavine hydrochloride delayed the DT of <u>L. monocytogenes</u> (by 0.4 h at 20 mg dm^{-3} and 11.0 h at 40 mg dm^{-3}), but did not adversely affect the quality of the electrical curves.

Using the routine conditions (see 2.3, 2.6 & 2.6.1) the impedimetric responses of mixed cultures of the test species used in the previous experiment were then monitored for 36 h in GNB supplemented with 0 to 40 mg dm^{-3} acriflavine hydrochloride. The results are tabulated (Table 4.20).

The impedance magnitude produced by the mixed culture was observed to depend upon the presence or absence of <u>L. monocytogenes</u>, with higher values being obtained when the target species was included. Extrapolation from data obtained with pure cultures (Table 4.19) would suggest that the impedimetric responses registered in the acriflavine hydrochloride supplemented GNB in the absence of <u>L. monocytogenes</u> were produced only by <u>Ps. fluorescens</u>. There was no evidence in this experiment of the impedance curves of <u>L. monocytogenes</u> being affected by the presence of non target species.

Further work was carried out to investigate the effects of a range of lower concentrations of acriflavine hydrochloride on an increased number of test microorganisms. The latter included a new culture of <u>L. monocytogenes</u> NCTC 11994, two additional serotypes of <u>L. monocytogenes</u> and closely related species <u>L. innocua</u>, <u>L. ivanovii</u> and <u>J. denitrificans</u>. Using the routine

Test species	Concentration of acriflavine hydrochloride ($mg\ dm^{-3}$)	Impedance DT (h) $\pm$ SD	Impedance magnitude (BUIC) $\pm$ SD
Mixed culture §	0	7.8$\pm$0.0	1040$\pm$ 16
+ L.	10	17.7$\pm$0.0	1054$\pm$ 49
monocytogenes	20	18.5$\pm$0.1	1027$\pm$ 2
NCTC 11994	40	19.0$\pm$0.8	1093$\pm$ 19
Mixed culture §	0	7.9$\pm$0.4	1013$\pm$ 14
- L.	10	20.6$\pm$1.2	568$\pm$ 23
monocytogenes	20	19.6$\pm$0.2	639$\pm$ 32
NCTC 11994	40	19.3$\pm$0.1	631$\pm$ 26

(N=2)

Key

§ = Each mixed culture consisted of:
Lact. lactis NCIB 6681,
Ent. faecalis NCIB 775,
Strep. salivarius NCIB 8883,
Lact. lactis subsp cremoris NCIB 8662,
Strep. agalactiae NCIB 8778,
Leuc. mesenteroides subsp cremoris NCIB 12008
and Ps. fluorescens NCIB 3756.

Table 4.20 The effect of acriflavine hydrochloride on the impedimetric responses of mixed cultures of test species.

conditions (see 2.3, 2.6 & 2.6.1) the impedimetric responses of these species were monitored for 36 h in GNB supplemented with 0 to 20 mg dm^{-3} acriflavine hydrochloride. The results are tabulated (Table 4.21).

It was determined that concentrations of acriflavine hydrochloride in excess of 10 mg dm^{-3} did not allow the impedimetric detection of all serotypes of L. monocytogenes, indicating that this is the highest concentration of agent that could be used in an

Test species	Concentration of acriflavine hydrochloride ($mg\ dm^{-3}$)	Impedance DT (h) ± SD	magnitude (BUIC) ± SD
L. monocytogenes NCTC 11994 (Old culture) Serotype 4b	0	14.1±3.9	1165± 40
	10	13.3±3.1	1208± 54
	15	17.2±4.4	1129±156
	20	22.9±2.7	938±122
L. monocytogenes NCTC 11994 (New culture) Serotype 4b	0	12.4±2.0	1265± 56
	10	13.5±3.0	1318±165
	15	20.9±3.3	1143±168
	20	29.9±5.2	80± 56 /
L. monocytogenes NCTC 7973 (Serotype 1a)	0	17.6±2.5	1208± 37
	10	30.8±0.9	440± 56
	15	> 36	0
	20	> 36	0
L. monocytogenes NCTC 5348 (Serotype 2)	0	11.0±2.0	1261± 46
	10	19.8±2.1	1333± 66
	15	25.4±1.6	1218±112
	20	> 36	0
L. innocua NCTC 10889	0	15.0±2.4	1199±109
	10	20.5±2.9	1267±138
	15	31.6±0.1	465±38
	20	> 36	0
L. ivanovii NCTC 11846	0	16.1±0.5	1165± 40
	10	> 36	0
	15	> 36	0
	20	> 36	0
J. denitrificans NCTC 10816	0	28.8±7.2	692±556
	10	> 36	0
	15	> 36	0
	20	> 36	0

(N=6)

Key

/ = Low impedance magnitude recorded as a result of a late DT preventing the entire electrical curve being registered within the assay time.

Table 4.21 The effect of acriflavine hydrochloride on the impedimetric responses of test species in GNB.

impedimetric assay for L. monocytogenes without the production of false negative results. This observation was not expected as the strain noted as most typical of the species, L. monocytogenes NCTC 11994 (NCTC Colindale, personal communication, 1990), was the most resistant of the listerial test species examined. This work suggests the members of the species Listeria have major differences in their tolerances to this toxic agent. Furthermore the impedimetric responses of L. ivanovii, which is also capable of instigating animal listeriosis, were inhibited at 10 mg dm^{-3}, the lowest concentration of acriflavine examined.

A 17.5 h increase in DT was obtained when the new strain of L. monocytogenes NCTC 11994 was grown in GNB supplemented with 20 mg dm^{-3} acriflavine hydrochloride, whereas the corresponding increase in the old culture was 8.8 h. This difference in resistance to the selective agent may be the result of the cells being in different physiological states, as the new culture was from a slope inoculated directly from the freeze dried vial within 24 h of its preparation.

Using the routine conditions (see 2.3, 2.6 & 2.6.1) the impedimetric responses of three lactic acid bacteria were monitored for 36 h in GNB supplemented with 0 to 20 mg dm^{-3} acriflavine hydrochloride. The results are tabulated (Table 4.22).

Test species	Concentration of acriflavine hydrochloride ($mg\ dm^{-3}$)	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
Lact.	0	11.4$\pm$0.5	789$\pm$ 22
lactis	10	> 36	0
NCIB 6681	15	> 36	0
	20	> 36	0
Ent.	0	8.8$\pm$0.5	540$\pm$ 17
faecalis	10	15.7$\pm$1.1	502$\pm$ 53
NCIB 775	15	> 36	0
	20	> 36	0
Strep.	0	7.5$\pm$0.1	559$\pm$ 24
salivarius	10	8.3$\pm$1.1	345$\pm$ 90
NCIB 8883	15	> 36	0
	20	> 36	0

(N=2)

Table 4.22 The effect of acriflavine hydrochloride on the impedimetric responses of three lactic acid bacteria.

The results showed that 10 mg dm^{-3} of acriflavine hydrochloride did not prevent the impedimetric detection of Ent. faecalis or Strep. salivarius. As L. monocytogenes NCTC 7973 was inhibited at 15 mg dm^{-3} (Table 4.21), this level of selective agent could not be used to selectively inhibit the detection of all lactic acid bacteria whilst allowing the detection of all L. monocytogenes strains.

It was concluded that if acriflavine hydrochloride were to be employed in a selective impedimetric medium it would need to be used in conjunction with other antimicrobial agents effective against non target Gram positive organisms.

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4.2.9 Investigation of proflavine hydrochloride as an agent selective for L. monocytogenes.

Acriflavine hydrochloride consists of two components, 3, 6-diaminoacridine (also known as proflavine hydrochloride) and 3, 6-diamino-10-methylacridium chloride. The former, which has not previously been used for the selective isolation of Listeria spp., was next evaluated. As proflavine hydrochloride is another fluorescein dye the spectrophotometric assay (see 2.5) was not used for this agent, but work was carried out to determine its effect on the electrical responses of Gram positive and Gram negative test species. Using routine conditions (see 2.3, 2.6 & 2.6.1) the impedimetric responses of four test species were monitored for 36 h in GNB supplemented with 0 to 21 mg dm^{-3} proflavine hydrochloride. The results are tabulated (Table 23).

L. monocytogenes produced an electrical curve in 21 mg dm^{-3} proflavine hydrochloride whereas the other Gram positive test species examined, Lact. lactis, was not detected at 7 mg dm^{-3}, the lowest concentration of proflavine used. The electrical responses of Gram negative E. coli were also inhibited by all concentrations of proflavine hydrochloride tested while those of Ps. fluorescens were unaffected by the antimicrobial agent. These results were expected as the same pattern had also occurred with acriflavine hydrochloride supplemented GNB (see 4.2.8).

Test species	Concentration of proflavine hydrochloride ($mg\ dm^{-3}$)	Impedance DT (h) ± SD	Impedance magnitude (BUIC) ± SD
L. monocytogenes NCTC 11994 (Serotype 4b)	0	11.1±3.5	1136±253
	7	12.3±3.2	1001± 71
	14	12.4±3.4	1142± 55
	21	13.7±3.5	1155±217
Lact. lactis NCIB 6681	0	9.0±2.3	993±388
	7	> 36	0
	14	> 36	0
	21	> 36	0
E. coli NCIB 86	0	8.6±1.2	668±139
	7	> 36	0
	14	> 36	0
	21	> 36	0
Ps. fluorescens NCIB 3756	0	7.7±1.9	879±121
	7	8.9±0.8	845± 90
	14	11.6±1.7	712±195
	21	15.6±5.7	718±156

(N=6)

Table 4.23 The effect of proflavine hydrochloride on the impedimetric responses of four test species.

As the acriflavine hydrochloride sensitivity of different serotypes of L. monocytogenes had been observed to vary (see Table 4.21), it was important to monitor the effect of proflavine hydrochloride on the electrical responses of a range of strains of the target organism. Using the routine conditions (see 2.3, 2.6 & 2.6.1) the impedimetric responses of four strains, comprising three serotypes of L. monocytogenes, were monitored for 36 h in GNB supplemented with 0 to 21 mg dm^{-3} of proflavine hydrochloride. The results are tabulated (Table 4.24).

Test species	Concentration of proflavine hydrochloride ($mg\ dm^{-3}$)	Impedance	
		DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
L.	0	16.6$\pm$2.0 #	866$\pm$ 63
monocytogenes	7	21.1$\pm$2.5	900$\pm$100
NCTC 7973	14	21.1$\pm$4.1	1155$\pm$101
(Serotype 1a)	21	23.0$\pm$6.5	840$\pm$262
L.	0	14.8$\pm$1.6	730$\pm$247
monocytogenes	7	19.2$\pm$1.9	742$\pm$ 26
NCTC 10357	14	20.2$\pm$2.5	1098$\pm$ 92
(Serotype 1a)	21	25.1$\pm$3.6	846$\pm$367
L.	0	11.1$\pm$0.5 #	800$\pm$198
monocytogenes	7	10.9$\pm$0.9	1017$\pm$165
NCTC 5348	14	12.8$\pm$0.7 #	1065$\pm$100
(Serotype 2)	21	14.7$\pm$1.1	1020$\pm$243
L.	0	11.3$\pm$0.7	1116$\pm$ 50
monocytogenes	7	14.4$\pm$2.6	1156$\pm$253
NCTC 10527	14	13.3$\pm$0.7	1351$\pm$133
(Serotype 4b)	21	17.4$\pm$2.4	1227$\pm$ 62

(N=6)

Key

= One artefactual curve produced

Table 4.24 The effect of proflavine hydrochloride on the impedimetric responses of different serotypes of L. monocytogenes.

All strains of L. monocytogenes examined produced a detection curve within 30 h in 21 mg dm^{-3} proflavine hydrochloride, with 16 h being the maximum delay in DT in the supplemented medium. Similar data were obtained with L. monocytogenes NCTC 11994 (Table 4.23). Furthermore very few (3 out of 96, 2 of these being with unsupplemented controls) artefactual curves were observed, with all remaining curves being of good quality and magnitude.

Further work was then carried out using the routine methods (see 2.3, 2.6 & 2.6.1) to determine the effect of a range of concentrations of proflavine hydrochloride on the impedimetric responses of a number of Listeria spp. and J. denitrificans. The results are tabulated in Table 4.25.

At a concentration of 21 mg dm^{-3} proflavine hydrochloride only the two strains of L. innocua and L. welshimeri produced detection curves within 36 h. The impedimetric responses of L. ivanovii were inhibited at concentrations in excess of 14 mg dm^{-3}, those of L. murrayi and L. seeligeri were inhibited at concentrations in excess of 7 mg dm^{-3}, while those of L. grayi and J. denitrificans were inhibited at 7 mg dm^{-3}, the lowest concentration examined.

Further work was carried out using the routine methods (see 2.3, 2.6 & 2.6.1) to determine the effect of a range of concentrations of proflavine hydrochloride on the impedimetric responses of ten listerial food isolates. The results of the experiments with two non haemolytic food isolates, I1 and I2, are given in Table 4.26, while the data obtained with eight haemolytic food isolates, I3 to I10, are given in Table 4.27.

Both of the non haemolytic listerial food isolates, identified as L. monocytogenes or L. innocua (see 2.1),

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Test species	Concentration of proflavine hydrochloride (mg dm^{-3})	Impedance DT (h) ± SD	Impedance magnitude (BUIC) ± SD
L. innocua NCTC 10889	0	13.5±1.6	1153±106
	7	15.7±1.0	976±160
	14	16.7±2.7	1150±144
	21	19.3±5.4	1019±206
L. innocua NCTC 11288	0	10.6±1.5	1173± 92
	7	14.0±4.4	1173± 18
	14	15.4±4.4	1037±365
	21	14.5±1.0	1242±120
L. welshimeri NCTC 11857	0	10.7±1.9	934±178
	7	11.4±0.5	1058± 43
	14	12.2±0.5 *	1283± 46
	21	14.6±3.6	1218±160
L. ivanovii NCTC 11846	0	16.2±2.1 #	636± 44
	7	22.5±4.2	660±150
	14	28.5±3.4	374±232
	21	> 36	0
L. murrayi NCTC 10812	0	16.9±0.9	1064±117
	7	30.8±3.8	1264±128
	14	> 36	0
	21	> 36	0
L. seeligeri NCTC 11856	0	16.6±2.0 #	1262±135
	7	18.7±1.1	955± 10
	14	> 36	0
	21	> 36	0
L. grayi NCTC 10815	0	12.8±1.2	985± 47
	7	> 36	0
	14	> 36	0
	21	> 36	0
J. denitrificans NCTC 10812	0	26.3±1.8	427±143
	7	> 36	0
	14	> 36	0
	21	> 36	0

(N=6)

Key
= One artefactual curve produced
* = Two artefactual curves produced

Table 4.25 The effect of proflavine hydrochloride on the impedimetric responses of Listeria spp. and J. denitrificans.

Non haemolytic Listeria food isolate	Concentration of proflavine hydrochloride $(mg\ dm^{-3})$	Impedance DT $(h) \pm SD$	magnitude $(BUIC) \pm SD$
I1	0	9.8 ± 0.8	916 ± 28
	7	12.3 ± 0.6 #	1179 ± 51
	14	14.9 ± 0.5	1009 ± 240
	21	13.2 ± 0.1	1191 ± 42
I2	0	10.1 ± 0.5	940 ± 80
	7	12.8 ± 1.0	1019 ± 232
	14	11.3 ± 0.2	981 ± 137
	21	11.5 ± 0.1 #	1247 ± 249

(N=4)

Key
= One artefactual curve produced

Table 4.26 The effect of proflavine hydrochloride on the impedimetric responses of non haemolytic food isolates (L. monocytogenes or L. innocua).

produced detection curves in all concentrations of proflavine hydrochloride examined. A maximum delay in DT of 3.4 h was observed at 21 mg dm^{-3}. Similarly all eight haemolytic food isolates, identified as L. monocytogenes, produced detection curves at all concentrations of proflavine hydrochloride examined. In this set of strains the greatest increase in DT in 21 mg dm^{-3} proflavine hydrochloride was observed to be 17.3 h.

Having shown that all strains of L. monocytogenes tested produced detection curves in GNB supplemented with

Haemolytic Listeria food isolate	Concentration of proflavine hydrochloride (mg dm^{-3})	Impedance	
		DT (h) ± SD	magnitude (BUIC) ± SD
I3	0	10.2±0.2	887± 13
	7	11.5±0.3	1103±115
	14	11.7±1.2 #	1047± 48
	21	13.6±1.0	1284±188
I4	0	9.4±0.7	942± 84
	7	10.8±0.4	1072±220
	14	11.8±0.6	990±125
	21	12.8±1.9	1144± 90
I5	0	10.0±0.1	910± 39
	7	10.7±0.3	1115±127
	14	12.9±0.3	1070±159
	21	15.6±0.5 #	1198±119
I6	0	8.4±0.7	944± 88
	7	10.7±0.5	1428± 41
	14	12.7±0.2	1072±250
	21	12.9±2.0	1161± 53
I7	0	9.6±0.1	968± 36
	7	11.1±0.1	1191±224
	14	11.8±0.3	1037±224
	21	12.1±0.5	1348±105
I8	0	7.9±0.6	970± 49
	7	9.9±0.2	1306±113
	14	12.4±0.3	962±234
	21	14.7±1.3	1262±119
I9	0	10.9±0.2	1097± 22
	7	12.6±0.2	971± 8
	14	18.3±3.1 #	1209±138
	21	28.2±2.2	921±206
I10	0	9.8±0.3	1098± 42
	7	11.2±0.3	1079± 42
	14	17.4±3.0	1021± 22
	21	25.7±1.6	1252± 69

(N=4)

Key
= One artefactual curve produced

Table 4.27 The effect of proflavine hydrochloride on the impedimetric responses of haemolytic food isolates (L. monocytogenes).

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21 mg dm^{-3} proflavine hydrochloride further work was executed using the routine conditions (see 2.3, 2.6 & 2.6.1) to determine the effect of a range of concentrations of this agent on the impedimetric responses of a spectrum of Gram positive test species. The results are tabulated in Table 4.28.

With the exception of B. cereus, the electrical responses of all the Gram positive test species were inhibited by 7 mg dm^{-3}, the lowest concentration of proflavine hydrochloride used. As B. cereus produced a detection curve in 14 mg dm^{-3} but not in 21 mg dm^{-3} proflavine hydrochloride, it was concluded that the latter concentration would be the minimum required to inhibit the detection curves of non target Gram positive microorganisms.

Further work was then undertaken using the routine methods (see 2.3, 2.6 & 2.6.1) to determine the effect of a range of concentrations of proflavine hydrochloride on the impedimetric responses of Gram negative test species. The results are tabulated in Table 4.29.

It was observed that while three pseudomonad species were resistant to 21 mg dm^{-3} proflavine hydrochloride (for data on Ps. fluorescens see Table 4.23), the electrical detection of E. coli was inhibited by 7 mg dm^{-3}, the lowest concentration tested (see Table 4.23).

Test species	Concentration of proflavine hydrochloride ($mg\ dm^{-3}$)	Impedance DT (h) $\pm$ SD	Impedance magnitude (BUIC) $\pm$ SD
Ent. faecalis NCIB 775	0	7.5$\pm$0.8	1150$\pm$132
	7	> 36	0
	14	> 36	0
	21	> 36	0
Strep. salivarius NCIB 8883	0	7.4$\pm$0.3	1259$\pm$ 96
	7	> 36	0
	14	> 36	0
	21	> 36	0
Lactob. casei subsp rhamnosus NCIB 6375	0	19.4$\pm$0.7	587$\pm$ 11
	7	> 36	0
	14	> 36	0
	21	> 36	0
M. roseus NCIB 8175	0	30.2$\pm$4.6	131$\pm$ 16
	7	> 36	0
	14	> 36	0
	21	> 36	0
B. cereus NCIB 8122	0	8.3$\pm$0.4	909$\pm$ 21
	7	12.7$\pm$4.1	711$\pm$207
	14	26.4$\pm$6.2	502$\pm$261
	21	> 36	0
B. circulans NCIB 7578	0	12.5$\pm$6.1	1103$\pm$ 67
	7	> 36	0
	14	> 36	0
	21	> 36	0

(N=6)

Table 4.28 The effect of proflavine hydrochloride on the impedimetric responses of non target Gram positive test species.

Test species	Concentration of proflavine hydrochloride ($mg\ dm^{-3}$)	Impedance	
		DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
Ps. putida NCIB 10936	0	7.2$\pm$0.3	516$\pm$ 34
	7	12.5$\pm$1.4	222$\pm$ 26
	14	20.6$\pm$1.4	373$\pm$128
	21	30.5$\pm$3.8	211$\pm$189
Ps. aeruginosa NCIB 950	0	9.4$\pm$1.7	798$\pm$492
	7	9.8$\pm$0.2	820$\pm$ 4
	14	12.1$\pm$0.7	384$\pm$ 78
	21	10.9$\pm$0.5	142$\pm$ 66
Ps. aeruginosa NCIB 10848	0	8.3$\pm$0.2 *	1983$\pm$ 80
	7	9.2$\pm$0.6	1446$\pm$260
	14	12.0$\pm$4.0	1488$\pm$140
	21	15.7$\pm$8.2	604$\pm$329

(N=4)

Key

* = Two artefactual curves produced

Table 4.29 The effect of proflavine hydrochloride on the impedimetric responses of Gram negative test species.

It was concluded that proflavine hydrochloride was the most promising anti Gram positive agent examined to date, but its lack of activity against pseudomonads would necessitate its use in combination with an anti Gram negative agent in a selective impedimetric assay for the target species.

4.3 Discussion - Individual Selective Agents

None of the selective agents examined possessed a broad enough spectrum of activity to allow the impedimetric detection of the target organism L. monocytogenes from a

range of test species expected to be present in food.

The selective agent which showed most promise in inhibiting the electrical responses of non target Gram positive organisms was proflavine hydrochloride. While this agent did not eliminate the detection curves of L. innocua and L. welshimeri, very importantly it did eliminate those produced by the closely related Gram positive Lactic acid bacteria. Furthermore proflavine hydrochloride did not adversely affect the morphology of the electrical curves produced by L. monocytogenes and in most strains 21 mg dm^{-3} delayed the DT by less than 10 h.

Two toxic agents, thallous acetate and moxalactam, were found to show an appropriate spectrum of activity in eliminating the detection curves produced by Gram negative microorganisms. As moxalactam is relatively non toxic to humans, cephalosporins are used therapeutically in medicine (Lietman, 1981), it would be used in preference to thallous acetate in an impedimetric assay.

It was next considered necessary to investigate combinations of antimicrobial agents with the aim of developing a selectively toxic medium capable of eliminating the detection curves of all non target Gram positive and Gram negative test species.

4.4 Results - Combinations of Selective Agents

A number of the selective agents which had been evaluated individually by impedimetric assay were also investigated in various combinations. The aim of this set of experiments was to broaden the spectrum of antimicrobial activity as no single agent had been found to be capable of eliminating the detection curves of all non _Listeria_ test species. The combinations of agents were evaluated for their spectrum of activity against a variety of Gram negative and Gram positive test species, possible synergism against _L. monocytogenes_ as well as for their influence on the electrical responses of the test microorganisms. Agents were investigated in pairs or larger groups as follows: thallous acetate and nalidixic acid (see 4.4.1), thallous acetate and moxalactam (see 4.4.2), thallous acetate and acriflavine hydrochloride (see 4.4.3), acriflavine hydrochloride and moxalactam (see 4.4.4), proflavine hydrochloride and moxalactam (see 4.4.5), proflavine hydrochloride, moxalactam, amphotericin B and ketoconazole (see 4.4.6), proflavine hydrochloride, moxalactam, aesculin and ammonium-ferric citrate (see 4.4.7) and proflavine hydrochloride, moxalactam and L-rhamnose (see 4.4.8)

4.4.1 Evaluation of the effect of a combination of thallous acetate and nalidixic acid on the impedimetric responses of test species.

The impedimetric responses of test species were

investigated in GNB supplemented with a variety of
concentrations of two anti Gram negative selective agents,
thallous acetate and nalidixic acid, with the aim of
determining whether these agents could act together to
produce anti Gram positive activity.

Using routine conditions (see 2.3, 2.6 & 2.6.1), the
impedimetric responses of four test species were monitored
for 36 h in GNB supplemented with a range of
concentrations of thallous acetate and nalidixic acid
(0 to 3 g dm^{-3} and 0 to 60 mg dm^{-3} respectively). Both
individual and mixed cultures were examined. The results
of this work are reported in Table 4.30.

The lowest concentration of antimicrobial agents
tested, 1 g dm^{-3} thallous acetate and 20 mg dm^{-3} nalidixic
acid, was inhibitory to the Gram negative test species,
Ps. fluorescens and E. coli. Unfortunately, addition of
this level of agents to GNB increased the DT of
L. monocytogenes by approximately 7 h which was far longer
than the corresponding increase in the DT of Lact. lactis
(Table 4.3o). A combination of 2 g dm^{-3} thallous acetate
and 40 mg dm^{-3} nalidixic acid was found to be inhibitory
to all the test microorganisms. As L. monocytogenes and
Lact. lactis produced detection curves in GNB supplemented
with either 3 g dm^{-3} thallous acetate (see 4.2.2) or
60 mg dm^{-3} nalidixic acid (see 4.2.1), this combination of
selective agents may be acting additively or
synergistically against both these Gram positive organisms
and therefore a selective advantage for the

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Test species	Concentration of thallous acetate (g dm^{-3})	nalidixic acid (mg dm^{-3})	DT (h) $\pm$ SD	Impedance magnitude (BUIC) $\pm$ SD
L.	0	0	9.4$\pm$0.3	895$\pm$ 6
monocytogenes	1	20	15.9$\pm$0.4	801$\pm$ 10
NCTC 11994	2	40	> 36	0
	3	60	> 36	0
Lact.	0	0	8.8$\pm$0.3	610$\pm$ 10
lactis	1	20	7.8$\pm$0.2	579$\pm$ 2
NCIB 6681	2	40	> 36	0
	3	60	> 36	0
E. coli	0	0	7.3$\pm$0.6	658$\pm$ 74
NCIB 86	1	20	> 36	0
	2	40	> 36	0
	3	60	> 36	0
Ps.	0	0	7.1$\pm$0.3	622$\pm$115
fluorescens	1	20	> 36	0
NCIB 3756	2	40	> 36	0
	3	60	> 36	0
Mixed culture $	0	0	6.4$\pm$0.0	943$\pm$ 15
+ L.	1	20	16.6$\pm$0.6	626$\pm$ 28
monocytogenes	2	40	> 36	0
	3	60	> 36	0
Mixed culture $	0	0	6.5$\pm$0.0	870$\pm$109
- L.	1	20	17.6$\pm$0.1	559$\pm$ 46
monocytogenes	2	40	> 36	0
	3	60	> 36	0

(N=2)

Key

$ = Each mixed culture consisted of:
Lact. lactis NCIB 6681,
E. coli NCIB 86 and
Ps. fluorescens NCIB 3756.

Table 4.30 The effect of a combination of thallous acetate and nalidixic acid on the impedimetric responses of individual test species and mixed cultures.

target species was not generated whilst using this combination of selective agents. There was little difference between the impedimetric responses of the mixed culture with or without L. monocytogenes, the target species could not be selectively detected using the combinations of antimicrobial agents investigated.

Work was carried out using routine conditions (see 2.3, 2.6 & 2.6.1) to determine whether thallous acetate at a fixed concentration of 3 g dm^{-3} in combination with various concentrations of nalidixic acid (0 to 21 mg dm^{-3}) would selectively inhibit the electrical responses of the lactic acid bacteria. 3 g dm^{-3} thallous acetate was used as it was found to be effective against Gram negative test species without increasing the DT of the target species L. monocytogenes unduly (Table 4.5). Up to 21 mg dm^{-3} nalidixic acid was used, as in the presence of 2 g dm^{-3} thallous acetate 40 mg dm^{-3} nalidixic acid was found to be inhibitory to all test species (Table 4.30). The results are tabulated in Table 4.31.

In this work a number of curves of uncharacteristic morphology were obtained (see Fig. 3.10 for examples of artefactual curves) making interpretation of the results difficult. However, it could be seen that this combination of selective agents did not allow the differentiation of L. monocytogenes from Lact. lactis as both test species have similar DT's.

Further combinations of these selective agents were not examined as from Table 4.30 it was determined increased concentrations required to improve selectivity

were inhibitory to the target species.

It was therefore concluded that at the concentrations tested the combination of thallous acetate and nalidixic acid was insufficiently selective for the sole detection of the target species L. monocytogenes. At lower concentrations, however, these agents might have a useful anti Gram negative role in a selective impedimetric medium.

Test species	Concentration of		Impedance	
	thallous acetate ($g\ dm^{-3}$)	nalidixic acid ($mg\ dm^{-3}$)	DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
	0	0	9.2$\pm$0.1	1037$\pm$ 35
L.	3	0	*	
monocytogenes	3	7	12.2$\pm$1.0	1025$\pm$ 25
NCTC 11994	3	14	13.4$\pm$1.8	1066$\pm$ 20
	3	21	13.8$\pm$0.5	753$\pm$ 46
	0	0	7.1$\pm$0.4	726$\pm$132
Lact.	3	0	11.4 #	593
lactis	3	7	11.4$\pm$0.1	599$\pm$ 5
NCIB 6681	3	14	13.7$\pm$0.4	715$\pm$ 13
	3	21	15.3$\pm$0.3	618$\pm$ 72
L.	3	0	*	
monocytogenes	3	7	13.4$\pm$0.1	499$\pm$ 19
+	3	14	13.8$\pm$0.0	900$\pm$237
Lact. lactis	3	21	12.9 #	665

(N=2)

Key
\# = One artefactual curve produced
* = Two artefactual curves produced

Table 4.31 The effect of a combination of thallous acetate and nalidixic acid on the impedimetric responses of L. monocytogenes and Lact. lactis in pure and mixed culture.

4.4.2 Evaluation of the effect of a combination of thallous acetate and moxalactam on the impedimetric responses of test species.

The impedimetric responses of test species were investigated in GNB supplemented with an anti Gram negative selective agent thallous acetate and the broad spectrum antibiotic moxalactam, with the aim of determining whether this combination of agents would permit the sole detection of L. monocytogenes.

Using routine conditions (see 2.3, 2.6 & 2.6.1), the impedimetric responses of eight test species were monitored for 36 h in GNB supplemented with a range of concentrations of thallous acetate (0 to 3 g dm^{-3}) and either 0 or 40 mg dm^{-3} moxalactam. 3 g dm^{-3} thallous acetate was used as it was found to be effective against Gram negative test species without increasing the DT of the target species L. monocytogenes unduly (Table 4.5). 40 mg dm^{-3} moxalactam was used as individually this concentration was found to inhibit the electrical responses of the standard three non Listeria test species without significant delay in the DT of L. monocytogenes (Table 4.10). The results are shown in Table 4.32.

Concentrations of the two agents which individually would not inhibit the impedimetric detection of L. monocytogenes (see 4.2.2 & 4.2.3) acted synergistically preventing the detection of the target species. Additionally, Ps. fluorescens and E. coli were inhibited. An electrical response of low magnitude was produced in

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Test species	Concentration of thallous acetate (g dm^{-3})	moxalactam (mg dm^{-3})	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
	0	0	9.5$\pm$0.1	1225$\pm$ 37
L.	0	40	11.5$\pm$0.0	1486$\pm$ 45
monocytogenes	1	40	> 36	0
NCTC 11994	2	40	> 36	0
	3	40	> 36	0
	0	0	7.2$\pm$0.3	625$\pm$ 21
Lact.	0	40	29.3 #	652
lactis	1	40	> 36	0
NCIB 6681	2	40	> 36	0
	3	40	> 36	0
	0	0	6.6$\pm$0.3	998$\pm$ 87
Ent.	0	40	6.7$\pm$0.3	1088$\pm$ 3
faecalis	1	40	8.3$\pm$0.1	1272$\pm$ 76
NCIB 775	2	40	9.3$\pm$0.4	1299$\pm$ 3
	3	40	9.8$\pm$0.1	1162$\pm$ 96
	0	0	6.1$\pm$0.3	958$\pm$ 76
Strep.	0	40	6.0$\pm$0.0	1025$\pm$ 8
agalactiae	1	40	7.2$\pm$0.1	1306$\pm$ 13
NCIB 8778	2	40	8.3$\pm$0.7	1205$\pm$ 31
	3	40	8.9$\pm$0.1	1171$\pm$ 24
	0	0	6.3$\pm$0.0	1011$\pm$ 92
Strep.	0	40	6.5$\pm$0.0	1043$\pm$ 13
salivarius	1	40	7.5$\pm$0.0	1213$\pm$ 1
NCIB 8883	2	40	9.7$\pm$0.0	1168$\pm$ 50
	3	40	9.7$\pm$0.1	1137$\pm$ 17
	0	0	29.1$\pm$3.8	249$\pm$ 6
Lactob.	0	40	?	122$\pm$ 2
casei	1	40	> 36	0
subsp rhamnosus	2	40	> 36	0
NCIB 6375	3	40	> 36	0

(Table 4.32 continued overleaf)

Test species	Concentration of		Impedance	
	thallous acetate (g dm^{-3})	moxalactam (mg dm^{-3})	DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
	0	0	8.1$\pm$0.7	670$\pm$ 36
E. coli	0	40	> 36	0
NCIB 86	1	40	> 36	0
	2	40	> 36	0
	3	40	> 36	0
	0	0	7.0$\pm$0.8	845$\pm$111
Ps.	0	40	> 36	0
fluorescens	1	40	> 36	0
NCIB 3756	2	40	> 36	0
	3	40	> 36	0

(N=2)

Key
? = DT's not determined
\# = One artefactual curve produced

Table 4.32 The effect of a combination of thallous acetate and moxalactam on the impedimetric responses of test species.

some reaction wells with Lactob. casei subsp rhamnosus but these were not allocated a DT by the Bactometer software. However, all lactic acid bacteria with the exception of Lact. lactis produced a detection curve at all concentrations tested.

Using routine conditions (see 2.3, 2.6 & 2.6.1), further work was undertaken to determine the effect of a combination of thallous acetate and moxalactam on the impedimetric responses of a variety of test species. In this set of experiments concentrations of thallous acetate remained constant at either 0 or 2 g dm^{-3} while moxalactam concentrations were varied from 0 to 60 mg dm^{-3}. These concentrations were used to confirm whether this

combination of selective agents acted additively or synergistically against the target species. The findings are tabulated in Table 4.33.

L. monocytogenes was not detected in any combination of the two agents, though it was detected in 2 g dm^{-3} thallous acetate alone. This was expected from previous work (Table 4.32) and confirmed that these selective agents act synergistically in the suppression of the impedimetric responses of L. monocytogenes. Additionally, all the lactic acid bacteria used except Lact. lactis were found to be resistant to the concentrations of agents tested. This variation in tolerance indicates that important cytological or biochemical differences do occur even within the same genus. Nevertheless the observed difference in tolerance between L. monocytogenes and most of the lactic acid bacteria tested suggests that the two genera may be separable in a selective impedimetric assay using appropriate antimicrobial agents. Neither the other Gram positive organism tested, Lactob. casei subsp rhamnosus, nor the Gram negative test species, E. coli and Ps. fluorescens, were detected in the presence of any combination of the selective agents.

It was therefore concluded that as the combination of thallous acetate and moxalactam was more inhibitory to L. monocytogenes than to certain other Gram positive test species it could not be used in a selective impedimetric detection regimen for L. monocytogenes. However, as few

Test species	Concentration of thallous acetate ($g\ dm^{-3}$)	moxalactam ($mg\ dm^{-3}$)	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
	0	0	8.1$\pm$0.3	1102$\pm$ 66
L.	2	0	9.2$\pm$0.1	1060$\pm$ 37
monocytogenes	2	20	> 36	0
NCTC 11994	2	40	> 36	0
	2	60	> 36	0
	0	0	7.1$\pm$0.3	558$\pm$ 16
Lact.	2	0	7.2$\pm$0.1	683$\pm$ 11
lactis	2	20	> 36	0
NCIB 6681	2	40	> 36	0
	2	60	> 36	0
	0	0	7.8$\pm$0.6	808$\pm$ 12
Strep.	2	0	8.4$\pm$0.2	905$\pm$ 31
agalactiae	2	20	8.0$\pm$0.3	856$\pm$ 33
NCIB 8778	2	40	8.3$\pm$0.0	962$\pm$ 12
	2	60	8.3$\pm$0.1	931$\pm$ 22
	0	0	7.7$\pm$0.1	967$\pm$ 69
Strep.	2	0	8.5$\pm$0.0	901$\pm$ 29
salivarius	2	20	8.6$\pm$0.1	1111$\pm$ 23
NCIB 8883	2	40	8.0$\pm$0.4	897$\pm$159
	2	60	7.9$\pm$0.3	1173$\pm$ 57
	0	0	28.7$\pm$4.8	231$\pm$ 43
Lactob.	2	0	> 36	0
casei	2	20	> 36	0
subsp rhamnosus	2	40	> 36	0
NCIB 6375	2	60	> 36	0
	0	0	8.7$\pm$0.0	707$\pm$ 63
E. coli	2	0	> 36	0
NCIB 86	2	20	> 36	0
	2	40	> 36	0
	2	60	> 36	0
	0	0	7.3$\pm$0.7	832$\pm$ 28
Ps.	2	0	> 36	0
fluorescens	2	20	> 36	0
NCIB 3756	2	40	> 36	0
	2	60	> 36	0

(N=2)

Table 4.33   The effect of a combination of thallous acetate and moxalactam on the impedimetric responses of test species.

artefactual curves were produced in this work, there is a possibility this combination of agents may have a useful curve stabilising effect.

4.4.3 Evaluation of the effect of a combination of thallous acetate and acriflavine hydrochloride on the impedimetric responses of test species.

The impedimetric responses of test species were investigated in GNB supplemented with an anti Gram negative selective agent thallous acetate and an anti Gram positive selective agent acriflavine hydrochloride, with the aim of determining whether this combination of agents would permit the sole detection of L. monocytogenes.

Using routine conditions (see 2.3, 2.6 & 2.6.1), the impedimetric responses of eight test organisms were monitored for 36 h in either unsupplemented GNB or GNB supplemented with 0.06 g dm^{-3} thallous acetate and 20 mg dm^{-3} acriflavine hydrochloride. The results of these experiments are tabulated (Table 4.34).

Addition of the selective agents to GNB inhibited the detection curves of all test species except L. monocytogenes. Furthermore the DT of the target species was not delayed at the concentration of selective agents used. Lact. lactis and Ps. fluorescens each produced one late detection curve out of six tests in the doubly supplemented medium; a false positive result for the detection regimen. On reinoculation from these reaction wells the organisms present were found to be sensitive to

Test species	Concentration of thallous acetate (g dm^{-3})	Concentration of acriflavine hydrochloride (mg dm^{-3})	Impedance DT (h) ± SD	Impedance magnitude (BUIC) ± SD
L. monocytogenes NCTC 11994	0	0 (N=2)	9.5±0.3	638± 21
	0.06	20 (N=6)	9.4±0.7	552± 24
Lact. lactis NCIB 6681	0	0 (N=2)	6.6±0.6	697±114
	0.06	20 (N=6)	20.8 (1) δ, > 36 (5) δ	550, 0
Ent. faecalis NCIB 775	0	0 (N=2)	7.8±0.1	682± 23
	0.06	20 (N=6)	> 36 #	0
Strep. salivarius NCIB 8883	0	0 (N=2)	7.1±0.0	547± 3
	0.06	20 (N=6)	> 36 #	0
B. cereus NCIB 8112	0	0 (N=2)	5.0±0.0	616± 21
	0.06	20 (N=6)	> 36	0
B. circulans NCIB 7578	0	0 (N=2)	11.5±0.0	409± 4
	0.06	20 (N=6)	> 36 #	0
B. subtilis NCIB 8703	0	0 (N=2)	12.5±0.1	381± 37
	0.06	20 (N=6)	> 36	0
Ps. fluorescens NCIB 3756	0	0 (N=2)	7.9±0.2	293± 50
	0.06	20 (N=6)	25.3(1) δ, > 36 (5) δ	360, 0

Key

\# = One artefactual curve produced

δ = Number of curves of this type in brackets

Table 4.34 The effect of a combination of thallous acetate and acriflavine hydrochloride on the impedimetric responses of test species.

the selective agents, indicating that airborne
contamination by resistant organisms was not the cause of
the false positives. These false positives were possibly
due to clumping of the test species in the dilution from
the overnight GNB culture (see 2.3), a greater number of
the test species being inoculated into the Bactometer
wells on these occasions.

In this set of experiments all test species
consistently produced impedance curves of low amplitude
both in the presence and absence of the selective agents.
The reasons for fluctuations in impedance magnitude are
discussed further later in this chapter (see 4.5).

It can be concluded that thallous acetate and
acriflavine hydrochloride show potential for use as
selective agents in an impedimetric assay for
L. monocytogenes. However, due to its human toxicity,
thallous acetate would not be used in preference to
another suitable anti Gram negative agent.

4.4.4 Evaluation of the effect of a combination of
acriflavine hydrochloride and moxalactam on the
impedimetric responses of test species.

The impedimetric responses of test species were
investigated in GNB supplemented with the anti Gram
positive selective agent acriflavine hydrochloride and the
broad spectrum agent moxalactam, with the aim of
determining whether this combination of agents would
permit the sole detection of L. monocytogenes.

Using routine procedures (see 2.3, 2.6 & 2.6.1), the impedimetric responses of eight test species were monitored for 36 h in GNB supplemented with 0 to 40 mg dm^{-3} acriflavine hydrochloride and either 0 or 20 mg dm^{-3} moxalactam. Both individual test species and mixed cultures were examined. The results of this work are reported in Table 4.35.

It was observed that 20 mg dm^{-3} acriflavine hydrochloride and 20 mg dm^{-3} moxalactam were the minimal concentrations of this combination of antimicrobial agents required to inhibit the detection of all non Listeria test species both individually and in mixed cultures. Furthermore, at these minimum effective concentrations, the DT and the quality of the electrical curves of L. monocytogenes were not affected.

Three lactic acid bacteria, Ent. faecalis, Strep. agalactiae and Strep. salivarius, produced detection curves in 10 mg dm^{-3} acriflavine hydrochloride and 20 mg dm^{-3} moxalactam. Importantly the DT's of the latter two organisms were only delayed 0.7 h and 0.3 h respectively. Hence a higher concentration of acriflavine hydrochloride, that is 20 mg dm^{-3}, was required to eliminate their electrical responses. These three lactic acid bacteria were therefore most likely to have been responsible for the detection curves produced by the mixed culture without L. monocytogenes in 10 mg dm^{-3} acriflavine hydrochloride and 20 mg dm^{-3} moxalactam.

Test species	Concentration of acriflavine hydrochloride (mg dm^{-3})	moxalactam (mg dm^{-3})	DT (h) $\pm$ SD	Impedance magnitude (BUIC) $\pm$ SD
_L._	0	0	11.3$\pm$0.3	663$\pm$ 5
monocytogenes	10	20	11.1$\pm$0.3	660$\pm$ 11
NCTC 11994	20	20	11.1$\pm$0.3	612$\pm$ 22
	40	20	12.6 #	610
Lact.	0	0	11.4$\pm$0.5	789$\pm$ 22
lactis	10	20	> 36	0
NCIB 6681	20	20	> 36	0
	40	20	> 36 #	0
Ent.	0	0	8.8$\pm$0.5	540$\pm$ 17
faecalis	10	20	17.3$\pm$0.0	416$\pm$ 6
NCIB 775	20	20	> 36	0
	40	20	> 36 #	0
Strep.	0	0	7.4$\pm$0.4	656$\pm$ 5
agalactiae	10	20	8.1$\pm$0.8	617$\pm$ 11
NCIB 8778	20	20	> 36 #	0
	40	20	> 36	0
Strep.	0	0	7.5$\pm$0.1	559$\pm$ 24
salivarius	10	20	7.8$\pm$0.0	512$\pm$ 1
NCIB 8883	20	20	> 36	0
	40	20	> 36	0
Lact. lactis	0	0	9.6$\pm$0.5	558$\pm$ 5
subsp _cremoris_	10	20	> 36 #	0
NCIB 8662	20	20	> 36 #	0
	40	20	> 36 #	0
Leuc.	0	0	14.2$\pm$0.1	941$\pm$ 25
mesenteroides	10	20	> 36	0
subsp _cremoris_	20	20	> 36	0
NCIB 12008	40	20	> 36	0
_Ps._	0	0	7.9$\pm$0.0	662$\pm$ 7
fluorescens	10	20	> 36	0
NCIB 3756	20	20	> 36	0
	40	20	> 36	0

(Table 4.35 continued overleaf)　　(N=2)

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| Test species | Concentration of | | Impedance | |
	acriflavine hydrochloride ($mg\ dm^{-3}$)	moxalactam ($mg\ dm^{-3}$)	DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
Mixed culture §	0	0	8.6 #	1075
+ <u>L.</u>	10	20	11.3$\pm$1.2	923$\pm$ 57
<u>monocytogenes</u>	20	20	14.2 #	839
NCTC 11994	40	20	> 36	0
Mixed culture §	0	0	7.7$\pm$0.3	899$\pm$153
− <u>L.</u>	10	20	9.3$\pm$1.6	489$\pm$137
<u>monocytogenes</u>	20	20	> 36	0
NCTC 11994	40	20	> 36	0

(N=2)

<u>Key</u>
§ = Each mixed culture consisted of:
<u>Lact. lactis</u> NCIB 6681,
<u>Ent. faecalis</u> NCIB 775,
<u>Strep. salivarius</u> NCIB 8883,
<u>Lact. lactis</u> subsp <u>cremoris</u> NCIB 8662,
<u>Strep. agalactiae</u> NCIB 8778,
<u>Leuc. mesenteroides</u> subsp <u>cremoris</u>
                                    NCIB 12008
and <u>Ps. fluorescens</u> NCIB 3756.
= One artefactual curve produced

Table 4.35 The effect of a combination of acriflavine hydrochloride and moxalactam on the impedimetric responses of individual test species and mixed cultures.

Further work was undertaken to determine the effect of 20 mg dm^{-3} acriflavine hydrochloride and 20 mg dm^{-3} moxalactam on an increased range of test species. The impedimetric responses of individual test species and mixed cultures were determined using the routine methods (see 2.3, 2.6 & 2.6.1). The results are tabulated (Table 4.36).

L. monocytogenes was the only monoculture to be detected in doubly supplemented GNB. Furthermore, the combination of 20 mg dm^{-3} acriflavine hydrochloride and 20 mg dm^{-3} moxalactam enabled the sole detection of L. monocytogenes from all four mixed cultures examined. Most importantly, there were no false negative detections from either the pure cultures or the mixed cultures containing L. monocytogenes (that is, L. monocytogenes was detected on all occasions). One false positive result was obtained with Ent. faecalis (out of the 16 reaction wells inoculated with this organism) and one false positive detection was produced by a mixed culture of lactic acid bacteria in the absence of L. monocytogenes (out of 8 reaction wells inoculated).

With the addition of the selective agents the DT of the L. monocytogenes monoculture increased approximately 50 %, from 9.6 to 14.3 h. This delay was not expected from previous work (Table 4.35). Statistical analysis (see 2.14) showed that this increase in DT was significant at a level of < 0.001 % (t = 10.98). Despite the fact that only L. monocytogenes was responsible for the detection curves produced in mixed cultures, statistical analysis showed

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Test species	Concentration of acriflavine hydrochloride ($mg\ dm^{-3}$)	moxalactam ($mg\ dm^{-3}$)	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
L. monocytogenes NCTC 11994	0 20	0 (N=8) 20 (N=16)	9.6$\pm$0.8 14.3$\pm$1.0 *	888$\pm$263 642$\pm$258
Lact. lactis NCIB 6681	0 20	0 (N=6) 20 (N=10)	8.6$\pm$0.9 > 36 #	623$\pm$154 0
Ent. faecalis NCIB 775	0 20	0 (N=8) 20 (N=16)	8.0$\pm$2.1 32.1 (1) &, # > 36 (14) &	708$\pm$238 262, 0 (15)
Strep. salivarius NCIB 8883	0 20	0 (N=6) 20 (N=8)	8.4$\pm$2.2 > 36	826$\pm$428 0
Strep. agalactiae NCIB 8778	0 20	0 (N=8) 20 (N=16)	9.7$\pm$2.4 # > 36 #	687$\pm$122 0
Leuc. mesenteroides subsp cremoris NCIB 12008	0 20	0 (N=6) 20 (N=8)	13.1$\pm$3.0 > 36 #	730$\pm$ 38 0
B. cereus NCIB 8112	0 20	0 (N=6) 20 (N=8)	6.0$\pm$0.3 > 36	1021$\pm$ 11 0
B. circulans NCIB 7578	0 20	0 (N=6) 20 (N=8)	10.7$\pm$0.3 > 36	994$\pm$154 0
B. subtilis NCIB 8703	0 20	0 (N=6) 20 (N=10)	8.7$\pm$2.2 > 36 #	694$\pm$ 64 0
E. coli NCIB 86	0 20	0 (N=6) 20 (N=10)	8.1$\pm$1.2 # > 36 #	719$\pm$212 0
Ps. fluorescens NCIB 3756	0 20	0 (N=6) 20 (N=10)	7.4$\pm$0.2 # > 36	600$\pm$175 0

(Table 4.36 continued overleaf)

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Test species	Concentration of acriflavine hydrochloride (mg dm^{-3})	moxalactam (mg dm^{-3})	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
Mixed culture 1 + L. monocytogenes NCTC 11994	20	20	14.2$\pm$4.0 *	1254$\pm$214
Mixed culture 1 - L. monocytogenes NCTC 11994	20	20	> 36 #	0
Mixed culture 2 + L. monocytogenes NCTC 11994	20	20	11.7$\pm$6.6 *	1199$\pm$446
Mixed culture 2 - L. monocytogenes NCTC 11994	20	20	24.5 (1) ♂, > 36 (7) ♂	1836, 0
Mixed culture 3 + L. monocytogenes NCTC 11994	20	20	20.7$\pm$2.5	981$\pm$192
Mixed culture 3 - L. monocytogenes NCTC 11994	20	20	> 36 #	0
Mixed culture 4 + L. monocytogenes NCTC 11994	20	20	27.0$\pm$4.3	709$\pm$ 45
Mixed culture 4 - L. monocytogenes NCTC 11994	20	20	> 36 #	0

Key   (N=8)

\# = One artefactual curve produced

* = Two artefactual curves produced

♂ = Number of curves of each type in brackets

Mixed Culture 1 = Lact. lactis NCIB 6681, Ent. faecalis NCIB 775, Strep. salivarius NCIB 8883, Strep. agalactiae NCIB 8778, B. subtilis NCIB 8703, E. coli NCIB 86 & Ps. fluorescens NCIB 3756.

Mixed Culture 2 = Lact. lactis NCIB 6681, Ent. faecalis NCIB 775, Strep. salivarius NCIB 8883 & Strep. agalactiae NCIB 8778.

Mixed Culture 3 = E. coli NCIB 86, Ps. fluorescens NCIB 3756 & B. subtilis NCIB 8703.

Mixed Culture 4 = Ent. faecalis NCIB 775, Strep. agalactiae NCIB 8778, E. coli NCIB 86 & Ps. fluorescens NCIB 3756.

Table 4.36 The effect of a combination of acriflavine hydrochloride and moxalactam on the impedimetric responses of individual test species and mixed cultures.

that significant differences in curve criteria occurred between the different mixed cultures. Tables 4.37 and 4.38 respectively illustrate the differences in DT's and impedance magnitudes. Mixed culture 4 (a combination of lactic acid bacteria and Gram negative species) produced the longest DT's and lowest impedance magnitudes of the four mixed cultures examined. The possible causes of these observed differences include: competition for nutrients

```
/-----------------------------------------------------------------\
| Mixed Culture    Mixed Culture    Mixed Culture    Mixed Culture |
|     1 + L.           2 + L.           3 + L.           4 + L.     |
| monocytogenes    monocytogenes    monocytogenes    monocytogenes |
|-----------------------------------------------------------------|
|       t = 0.65 ( > 20 % )                                       |
|          |--------------|                                       |
|                                                                 |
|                                                                 |
|              t = 3.14 ( < 2 % )                                 |
|          |-----------------------------|                       |
|                                                                 |
|                                                                 |
|              t = 4.87 ( < 0.001 % )                             |
|          |------------------------------------------|          |
|                                                                 |
|                                                                 |
|                  t = 2.99 ( < 0.01 % )                          |
|                 |---------------|                               |
|                                                                 |
|                                                                 |
|                  t = 4.60 ( < 0.001 % )                         |
|                 |-----------------------------|                 |
|                                                                 |
|                                                                 |
|                      t = 3.07 ( < 0.01 % )                      |
|                      |----------------|                         |
|                                                                 |
|                                                                 |
\-----------------------------------------------------------------/
```

Table 4.37 Statistical analysis of the DT's produced by L. monocytogenes in the four mixed cultures. Values of t are given for each pair of mixed cultures and the corresponding significance levels are shown in brackets.

```
/--------------------------------------------------------------\
| Mixed Culture     Mixed Culture     Mixed Culture     Mixed Culture |
|     1 + L.            2 + L.            3 + L.            4 + L.   |
| monocytogenes     monocytogenes     monocytogenes     monocytogenes |
|--------------------------------------------------------------------|
|        t = 0.22 ( > 20% )                                          |
|            |--------------|                                        |
|                                                                    |
|                                                                    |
|            t = 2.11 ( < 10 % )                                     |
|            |------------------------------|                        |
|                                                                    |
|                                                                    |
|              t = 4.98 ( < 0.001 % )                                |
|            |------------------------------------------------|      |
|                                                                    |
|                                                                    |
|                  t = 1.05 ( > 20 % )                               |
|                  |---------------|                                 |
|                                                                    |
|                                                                    |
|                    t = 2.18 ( < 10 % )                             |
|                  |------------------------------|                  |
|                                                                    |
|                                                                    |
|                      t = 2.82 ( < 2 % )                            |
|                        |----------------|                          |
|                                                                    |
|                                                                    |
\--------------------------------------------------------------/
```

Table 4.38 Statistical analysis of the impedance
magnitudes produced by L. monocytogenes in the four mixed
cultures. Values of t are given for each pair of mixed
cultures and the corresponding significance levels are
shown in brackets.

prior to the selective agents taking effect, endotoxin

release from Gram negative species affected by toxic

agents, and production of bacteriocins. In addition the

mixed cultures were observed to produce a high proportion

of artefactual curves.

Additional experiments were carried out to determine

the minimum concentration of L. monocytogenes detectable

by impedimetric assay in 20 mg dm^{-3} acriflavine

hydrochloride and 20 mg dm^{-3} moxalactam supplemented GNB. Using routine methods (see 2.3, 2.4, 2.6, 2.6.1 & 2.6.2) serially diluted samples of L. monocytogenes NCTC 11994 were simultaneously monitored for TVC, DT and impedance magnitude. The results are tabulated (Table 4.39) and shown graphically (Fig. 4.1).

Throughout the range of inocula used L. monocytogenes was detected by impedimetric assay in the doubly supplemented GNB. The DT increased with decreasing numbers of organisms whilst mean impedance magnitude remained relatively constant. A significant negative correlation ($r = - 0.9408$, $p = 0.05$ %) was observed between inoculum concentration and DT (Fig. 4.1). Using this relationship it would therefore be possible to simultaneously detect and enumerate monocultures of L. monocytogenes in GNB supplemented with acriflavine hydrochloride and moxalactam.

An inoculum of 10^4 CFU of L. monocytogenes yielded a mean DT of 9.2 h in unsupplemented GNB and a mean DT of 10.2 h in GNB supplemented with 20 mg dm^{-3} acriflavine hydrochloride and 20 mg dm^{-3} moxalactam (Table 4.39). Statistical analysis (see 2.14) showed that this delay in DT was significant at the 5 % level ($t = 2.43$), and therefore use of these selective agents reduced the rapidity of the assay. However, the selective agents were not found to have any significant influence on the magnitude of the impedance curves produced by the 10^4 CFU inoculum of L. monocytogenes (mean magnitude was 799 BUIC in unsupplemented medium and 772 BUIC in doubly

TVC of inoculum (CFU cm^{-3} ± SD)	Concentration of acriflavine hydrochloride (mg dm^{-3})	moxalactam (mg dm^{-3})	Impedance DT (h) ± SD	magnitude (BUIC) ± SD
First experiment (N=6)				
Control				
$1.0 \pm 0.1 \times 10^4$	0	0	9.2±0.7	632± 68
$1.0 \pm 0.1 \times 10^7$	20	20	4.4±0.2	592± 28
$1.0 \pm 0.1 \times 10^6$	20	20	6.6±0.3	603± 50
$1.0 \pm 0.1 \times 10^5$	20	20	7.2±1.1	595± 89
$1.0 \pm 0.1 \times 10^4$	20	20	10.1±0.7	569± 93
$1.0 \pm 0.1 \times 10^3$	20	20	13.0±0.8 #	612± 91
$1.0 \pm 0.1 \times 10^2$	20	20	14.7±1.6	590± 96
$1.0 \pm 0.1 \times 10^1$	20	20	18.3±0.9 #	585± 98
Second experiment (N=6)				
Control				
$1.5 \pm 0.5 \times 10^4$	0	0	9.2±0.6 #	999± 28
$1.5 \pm 0.5 \times 10^7$	20	20	3.5±0.1	979± 52
$1.5 \pm 0.5 \times 10^6$	20	20	6.1±0.0	992± 87
$1.5 \pm 0.5 \times 10^5$	20	20	7.5±0.6	991± 90
$1.5 \pm 0.5 \times 10^4$	20	20	10.2±1.4	975± 37
$1.5 \pm 0.5 \times 10^3$	20	20	12.0±0.5 Ç	1161± 27
$1.5 \pm 0.5 \times 10^2$	20	20	14.8±2.3	1021±104
$1.5 \pm 0.5 \times 10^1$	20	20	14.4±3.2 #	1088± 82
Averaged data for the 10^4 CFU inoculum from the two experiments (N=12)				
Control				
$1.3 \pm 0.5 \times 10^4$	0	0	9.2±0.6 #	799±190
$1.3 \pm 0.5 \times 10^4$	20	20	10.2±1.1	772±215

Key
\# = One artefactual curve produced
* = Two artefactual curves produced
Ç = Three artefactual curves produced

Table 4.39 Impedimetric responses of a range of inoculum concentrations of **L. monocytogenes** NCTC 11994 in 20 mg dm^{-3} acriflavine hydrochloride and 20 mg dm^{-3} moxalactam supplemented GNB.
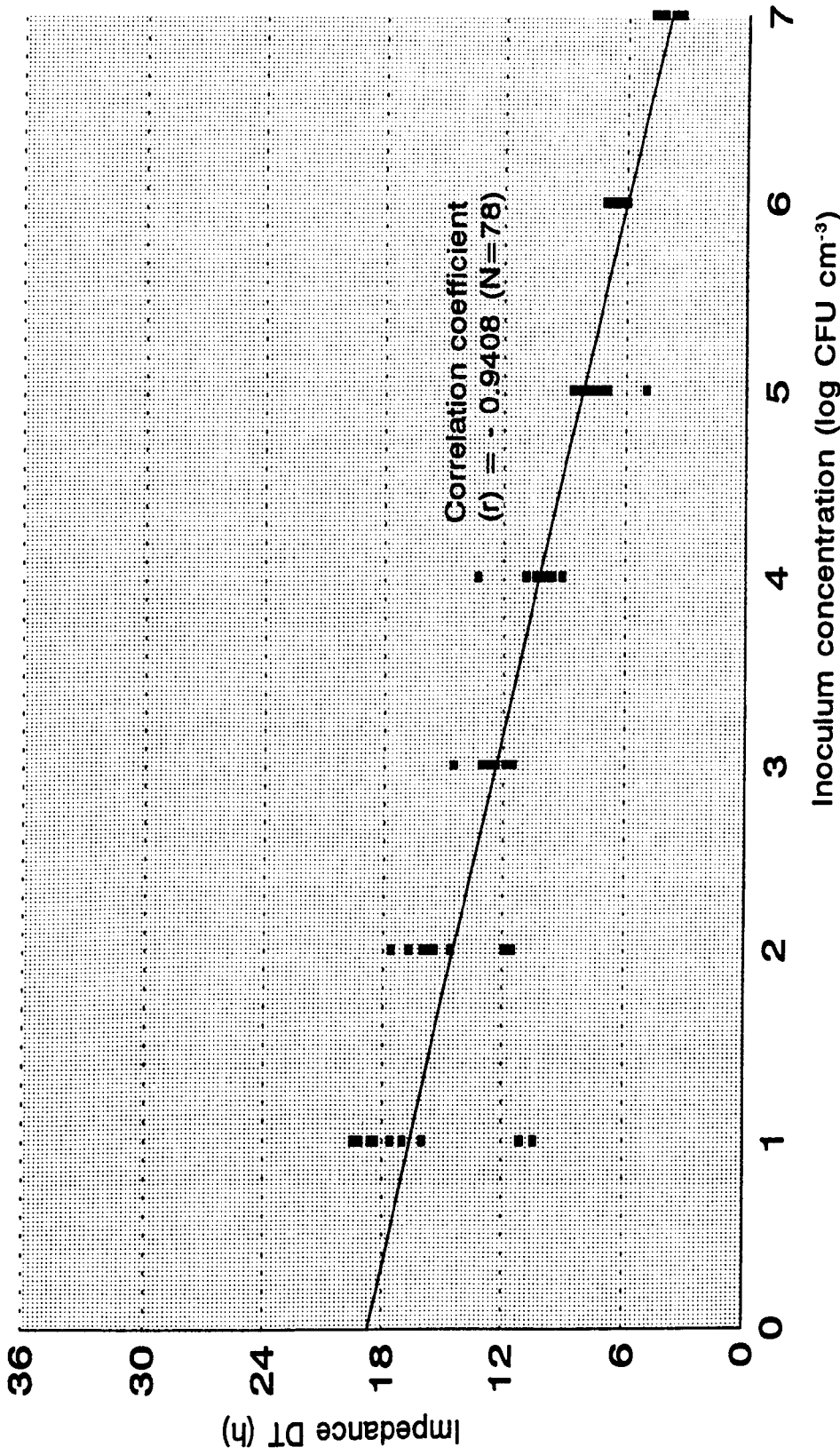
Fig. 4.1 Relationship between inoculum concentration and DT of L. monocytogenes
NCTC 11994 in 20 mg dm^{-3} acriflavine hydrochloride and 20 mg dm^{-3} moxalactam
supplemented GNB (pooled data from first and second experiment).

supplemented medium; t = 0.30, p = > 20 %).

To investigate the reproducibility of the impedimetric responses of <u>L. monocytogenes</u> in the doubly supplemented GNB data obtained using a 10^4 CFU inoculum on two separate occasions (see Table 4.39) were compared using the t-Test (see 2.14). The mean DT in the first assay was 10.1 h, whilst that in the second assay was 10.2 h. This difference is not significant (t = 0.39, p = > 20 %). However, the mean magnitude of the first assay was 569 BUIC, whilst that in the second assay was 975 BUIC. This difference is significant at the 0.001 % level (t = 9.01). These results indicate that a detection regimen for <u>L. monocytogenes</u> should not utilise impedimetric magnitude as a curve criterion as significant inter-experiment differences occur. Variation in electrical response is discussed further later in this chapter (see 4.5).

The effect of the combination of acriflavine hydrochloride and moxalactam was next investigated on an extended range of test organisms including a second strain of <u>L. monocytogenes</u> and a number of closely related species as well as an increased number of Gram negative organisms. The impedimetric responses of these species individually and in mixed culture were determined using routine methods (see 2.3, 2.6 & 2.6.1). The results are tabulated (Table 4.40).

No individual test species except <u>L. monocytogenes</u> NCTC 11994 was consistently detected in 20 mg dm^{-3} acriflavine hydrochloride and 20 mg dm^{-3} moxalactam

supplemented GNB. As this strain was detected with only a small increase in DT in the doubly supplemented medium compared with the unsupplemented medium the failure to detect L. monocytogenes NCTC 10357 was not expected. The known difference in serotype between these two strains, and hence the presumed difference in cell surface structure, may account for this observed difference in their susceptibility to the selective agents. As all known serotypes of L. monocytogenes are potentially pathogenic the detection of all is equally important. A second unexpected result in this set of experiments was the production of a detection curve in the doubly supplemented medium by two mixed cultures not containing L. monocytogenes. However, the addition of L. monocytogenes resulted in a decrease in DT in both cases. Assuming that the initial concentration of antimicrobial agents in the medium is reduced as a result of uptake by growing cells, then the presence of a greater number of organisms in mixed cultures (where the inoculum consists of 0.1 cm^3 of a 10^{-4} dilution of each test species) than in pure cultures (where the inoculum consists of 0.1 cm^3 of a 10^{-4} dilution of one test species) may require GNB to be supplemented with higher concentrations of the selective agents in order to maintain active concentrations in solution and permit differentiation of the target species.

Test species	Concentration of acriflavine hydrochloride (mg dm^{-3})	moxalactam (mg dm^{-3})	Impedance DT (h) ± SD	magnitude (BUIC) ± SD
L. monocytogenes NCTC 10357 (Serotype 1a)	0	0	12.2±2.5	1183± 74
	20	20	> 36	0
L. monocytogenes NCTC 11994 (Serotype 4b)	0	0	9.9±1.6	1253±145
	20	20	14.9±2.3	1233±102
L. ivanovii NCTC 11846	0	0	13.2±0.8	954±108
	20	20	> 36	0
L. innocua NCTC 10889	0	0	10.6±0.5	1191± 92
	20	20	> 36	0
L. innocua NCTC 11288	0	0	10.3±0.4	1104±145
	20	20	31.0±1.1 (3) ð	523±109
			> 36 (2) ð	0
L. seeligeri NCTC 11856	0	0	13.5±3.0 #	1351± 38
	20	20	> 36 #	0
J. denitrificans NCTC 10816	0	0	20.5±7.1	654±192
	20	20	> 36 *	0
M. roseus NCIB 8175	0	0	> 36 #	0
	20	20	> 36	0
M. luteus NCIB 9278	0	0	> 36	0
	20	20	> 36	0

(Table 4.40 continued overleaf)   (N=6)

Test species	Concentration of acriflavine hydrochloride ($mg\ dm^{-3}$)	moxalactam ($mg\ dm^{-3}$)	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
Ps. aeruginosa NCIB 950	0 20	0 20	8.7$\pm$1.6 > 36 *	1253$\pm$253 0
Ps. aeruginosa NCIB 10848	0 20	0 20	7.7$\pm$0.8 > 36	1699$\pm$424 0
Ps. putida NCIB 10936	0 20	0 20	6.6$\pm$0.9 > 36 *	690$\pm$250 0
E. coli NCIB 86	0 20	0 20	7.5$\pm$0.2 > 36	732$\pm$ 94 0
Mixed culture 1 + L. monocytogenes NCTC 11994	0 20	0 20	8.8$\pm$0.5 12.0$\pm$0.3	1148$\pm$ 23 1175$\pm$ 21
Mixed culture 1 - L. monocytogenes NCTC 11994	0 20	0 20	8.8$\pm$0.6 30.9$\pm$2.8	1156$\pm$85 195$\pm$241
Mixed culture 2 + L. monocytogenes NCTC 11994	0 20	0 20	4.8$\pm$0.1 9.7$\pm$0.5	1301$\pm$ 15 1230$\pm$ 84
Mixed culture 2 - L. monocytogenes NCTC 11994	0 20	0 20	5.2$\pm$0.1 15.6$\pm$1.1	1329$\pm$ 13 1046$\pm$273

(N=6)

Key

= One artefactual curve produced

* = Two artefactual curves produced

δ = Number of curves of this type in brackets

Mixed Culture 1 = L. ivanovii NCTC 11846, L. innocua NCTC 10889, L. innocua NCTC 11288, L. seeligeri NCTC 11856, J. denitrificans NCTC 10816.

Mixed Culture 2 = L. ivanovii NCTC 11846, L. innocua NCTC 10889, L. innocua NCTC 11288, L. seeligeri NCTC 11856, J. denitrificans NCTC 10816, M.roseus NCIB 8175, M.luteus NCIB 9278, Ps. aeruginosa NCIB 950, Ps. aeruginosa NCIB 10848, Ps. putida NCIB 10936.

Table 4.40 The effect of a combination of acriflavine hydrochloride and moxalactam on the impedimetric responses of individual test species and mixed cultures.

Further work was then undertaken to examine the effect of acriflavine hydrochloride and moxalactam on four different strains of L. monocytogenes of varying serotype. The impedimetric responses of these organisms were determined using routine methods (see 2.3, 2.6 & 2.6.1). The results are tabulated (Table 4.41).

L. monocytogenes NCTC 11994 was the only strain to be detected on all occasions. The concentration of selective agents used severely delayed or inhibited detection of all the other strains of L. monocytogenes examined.

Test species	Concentration of acriflavine hydrochloride ($mg\ dm^{-3}$)	moxalactam ($mg\ dm^{-3}$)	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
L. monocytogenes NCTC 7973 (Serotype 1a)	0 20	0 20	13.9$\pm$0.4 34.5 (1) ẟ, * > 36 (3) ẟ	1067$\pm$ 56 159, 0
L. monocytogenes NCTC 5348 (Serotype 2)	0 20	0 20	9.7$\pm$0.6 30.1$\pm$3.4 (2) ẟ, # > 36 (3) ẟ	1130$\pm$157 198$\pm$ 41 0
L. monocytogenes NCTC 11994 (Serotype 4b)	0 20	0 20	10.2$\pm$0.5 # 21.6$\pm$2.4 *	1199$\pm$ 64 793$\pm$266
L. monocytogenes NCTC 10527 (Serotype 4b)	0 20	0 20	9.7$\pm$0.3 34.0 (1) ẟ, > 36 (5) ẟ	1258$\pm$ 46 103, 0

(N=6)

<u>Key</u>
= One artefactual curve produced
* = Two artefactual curves produced
ẟ = Number of curves of this type in brackets

Table 4.41 The effect of a combination of acriflavine hydrochloride and moxalactam on the impedimetric responses of various strains of L. monocytogenes.

The effect of a combination of acriflavine hydrochloride and moxalactam on ten Listeria food isolates (see 2.1) was next investigated. The impedimetric responses of these food isolates were determined using the routine methods (see 2.3, 2.6 & 2.6.1). The results are tabulated in Table 4.42.

As in previous experiments L. monocytogenes NCTC 11994 (control) was consistently detected. However, 20 mg dm^{-3} acriflavine hydrochloride and 20 mg dm^{-3} moxalactam was found to be too inhibitory to allow the reliable detection of any of the Listeria food isolates.

It was therefore concluded that supplementation of GNB with 20 mg dm^{-3} acriflavine hydrochloride and 20 mg dm^{-3} moxalactam affected the electrical responses of a range of test bacteria, the acriflavine hydrochloride eliminating non Listeria Gram positive test species (see 4.2.8) and the moxalactam eliminating Gram negative pseudomonad species (see 4.2.5). However, at this level of selective agents, detection curves were produced by some mixed cultures of test species in the absence of L. monocytogenes (Table 4.40). It would not be feasible, therefore, to use lower concentrations of selective agents to allow detection of the strains of L. monocytogenes which were inhibited by the present levels (Tables 4.40, 4.41 & 4.42). An alternative anti Gram positive agent is obviously required which is less toxic to L. monocytogenes strains and more toxic to non Listeria organisms. As lactic acid bacteria did not produce detection curves in

Test species	Concentration of acriflavine hydrochloride (mg dm^{-3})	moxalactam (mg dm^{-3})	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
L. monocytogenes NCTC 11994	0	0	11.6$\pm$2.2	718$\pm$198
	20	20	19.4$\pm$1.1	606$\pm$549
Food isolate I1	0	0	10.7$\pm$1.2	732$\pm$ 94
	20	20	29.3$\pm$0.5 (3) ᵟ, > 36 (3) ᵟ	1100$\pm$ 68 0
Food isolate I2	0	0	10.1$\pm$1.9	751$\pm$163
	20	20	29.1$\pm$0.8 (3) ᵟ, > 36 (3) ᵟ	759$\pm$369 0
Food isolate I3	0	0	9.2$\pm$0.1	875$\pm$212
	20	20	> 36 *	0
Food isolate I4	0	0	10.4$\pm$1.6	811$\pm$211
	20	20	> 36	0
Food isolate I5	0	0	12.0$\pm$1.7 #	678$\pm$237
	20	20	> 36	0
Food isolate I6	0	0	11.9$\pm$1.3	550$\pm$241
	20	20	> 36	0
Food isolate I7	0	0	13.7$\pm$3.9	749$\pm$ 280
	20	20	> 36 #	0
Food isolate I8	0	0	11.3$\pm$1.6	724$\pm$139
	20	20	> 36 #	0
Food isolate I9	0	0	10.4$\pm$0.1 #	888$\pm$ 21
	20	20	> 36	0
Food isolate I10	0	0	9.6$\pm$0.1	957$\pm$ 38
	20	20	34.6 (1) ᵟ, > 36 (5) ᵟ	127, 0

<u>Key</u>   (N=6)
\# = One artefactual curve produced
* = Two artefactual curves produced
ᵟ = Number of curves of each type in brackets

Table 4.42 The effect of a combination of acriflavine hydrochloride and moxalactam on the impedimetric responses of <u>Listeria</u> food isolates.

7 mg dm^{-3} proflavine hydrochloride while all strains of
L. monocytogenes examined were detected at 21 mg dm^{-3} (see
4.2.9), this agent could be a useful alternative to
acriflavine hydrochloride. Its toxic spectrum in
combination with moxalactam was therefore next evaluated.

4.4.5 Evaluation of the effect of a combination of
proflavine hydrochloride and moxalactam on the
impedimetric responses of test species.

The impedimetric responses of test species were
investigated in GNB supplemented with the anti Gram
positive selective agent proflavine hydrochloride and the
broad spectrum agent moxalactam, with the aim of
determining whether this combination of agents would
permit the sole detection of L. monocytogenes.

Using routine conditions (see 2.3, 2.6 & 2.6.1) the
impedimetric responses of four test species were monitored
for 36 h in either unsupplemented GNB or GNB supplemented
with 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3}
moxalactam. The results are tabulated in Table 4.43.

Of the test species examined only L. monocytogenes
NCTC 11994 produced detection curves in the doubly
supplemented GNB. These curves were of good morphology and
magnitude. This combination of selective agents was
therefore judged suitable for further examination.

Work was then carried out using routine conditions
(see 2.3, 2.6 & 2.6.1) to determine the impedimetric
responses of an increased range of Gram positive and
negative test species in either unsupplemented GNB or GNB

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| Test species | Concentration of | | Impedance | |
	proflavine hydrochloride (mg dm^{-3})	moxalactam (mg dm^{-3})	DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
L.	0	0	9.3$\pm$0.3	1064$\pm$129
monocytogenes	21	20	13.1$\pm$1.8	1012$\pm$ 21
NCTC 11994				
Lact.	0	0	9.9$\pm$0.4	762$\pm$132
lactis	21	20	> 36	0
NCIB 6681				
Ps.	0	0	10.1$\pm$0.1	497$\pm$187
fluorescens	21	20	> 36	0
NCIB 3756				
E.	0	0	7.6$\pm$0.3	539$\pm$135
coli	21	20	> 36	0
NCIB 86				

(N=6)

Table 4.43 The effect of a combination of proflavine hydrochloride and moxalactam on the impedimetric responses of test species.

supplemented with 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam. The results are presented in Table 4.44.

L. monocytogenes NCTC 11994 and L. monocytogenes food isolate I4 (see 2.1) were the only test species that produced a detection curve within 36 h in the doubly supplemented GNB. The inhibition of the Gram positive lactic acid bacteria is of significant importance. Such organisms are both common contaminants and members of the starter inocula of dairy products and, as they are related to L. monocytogenes, were expected to be the most difficult non target organisms to eliminate in a selective impedimetric assay.

Test species	Concentration of proflavine hydrochloride ($mg\ dm^{-3}$)	moxalactam ($mg\ dm^{-3}$)	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
L. monocytogenes NCTC 11994	0	0	8.7$\pm$0.6	947$\pm$ 79
	21	20	11.4$\pm$0.8	1117$\pm$ 61
L. monocytogenes food isolate I4	0	0	8.8$\pm$0.1	1033$\pm$ 7
	21	20	15.3$\pm$1.0	970$\pm$ 71
Lact. lactis NCIB 6681	0	0	7.9$\pm$1.1	882$\pm$ 52
	21	20	> 36 h	0
Ent. faecalis NCIB 775	0	0	6.0$\pm$0.3	1015$\pm$ 5
	21	20	> 36 h	0
Strep. salivarius NCIB 8883	0	0	7.4$\pm$0.3	1259$\pm$ 96
	21	20	> 36 h	0
Lactob. casei subsp rhamnosus NCIB 6375	0	0	19.4$\pm$0.7	587$\pm$ 11
	21	20	> 36 h	0
M. roseus NCIB 8175	0	0	18.9$\pm$0.9	905$\pm$ 57
	21	20	> 36 h	0
B. cereus NCIB 8122	0	0	8.3$\pm$0.4	909$\pm$ 21
	21	20	> 36 h	0
B. circulans NCIB 7578	0	0	12.5$\pm$6.1	1103$\pm$ 67
	21	20	> 36 h	0
B. subtilis NCIB 8703	0	0	11.4$\pm$0.5	707$\pm$ 1
	21	20	> 36 h	0

(Table 4.44 continued overleaf)   (N=6)

Test species	Concentration of proflavine hydrochloride ($mg\ dm^{-3}$)	moxalactam ($mg\ dm^{-3}$)	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
Ps. fluorescens NCIB 3756	0 21	0 20	21.3$\pm$0.1 > 36 h	288$\pm$ 9 0
Ps. putida NCIB 10936	0 21	0 20	6.9$\pm$0.6 > 36 h	428$\pm$ 99 0
Ps. aeruginosa NCIB 950	0 21	0 20	8.9$\pm$0.7 > 36 h	547$\pm$312 0
Ps. aeruginosa NCIB 10848	0 21	0 20	9.5$\pm$0.2 > 36 h	1409$\pm$ 50 0
E. coli NCIB 86	0 21	0 20	8.6$\pm$0.2 > 36 h	561$\pm$ 15 0

(N=6)

Table 4.44 The effect of a combination of proflavine hydrochloride and moxalactam on the impedimetric responses of test species.

Using routine conditions (see 2.3, 2.6 & 2.6.1) the impedimetric responses of four L. monocytogenes strains and a number of closely related species were monitored for 36 h in either unsupplemented GNB or GNB supplemented with 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam. The results of this work are reported in Table 4.45. An investigation of the impedimetric responses of a variety of mixed cultures in the doubly supplemented medium was also undertaken and the results presented in Table 4.45. The corresponding control values obtained with

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Test species	Concentration of proflavine hydrochloride (mg dm^{-3})	moxalactam (mg dm^{-3})	Impedance DT $(h) \pm SD$	magnitude $(\text{BUIC}) \pm SD$
L. *monocytogenes* NCTC 7973 (Serotype 1a)	0 21	0 20	9.8 ± 1.1 14.8 ± 1.2	948 ± 93 1037 ± 125
L. *monocytogenes* NCTC 10357 (Serotype 1a)	0 21	0 20	8.9 ± 0.6 27.6 ± 0.8 *	1092 ± 38 970 ± 34
L. *monocytogenes* NCTC 5348 (Serotype 2)	0 21	0 20	12.0 ± 1.0 20.7 ± 0.1	1214 ± 57 1001 ± 14
L. *monocytogenes* NCTC 10527 (Serotype 4b)	0 21	0 20	8.9 ± 0.9 19.7 ± 3.1 #	1093 ± 98 1146 ± 182
L. *ivanovii* NCTC 11846	0 21	0 20	19.5 ± 0.3 > 36	861 ± 41 0
L. *innocua* NCTC 10889	0 21	0 20	12.4 ± 3.3 25.7 ± 1.6	1234 ± 106 531 ± 7
L. *innocua* NCTC 11288	0 21	0 20	13.5 ± 0.7 15.1 ± 0.6 #	1297 ± 61 1253 ± 115
L. *welshimeri* NCTC 11857	0 21	0 20	16.4 ± 8.1 27.8 ± 1.9	889 ± 184 1034 ± 329
L. *seeligeri* NCTC 11856	0 21	0 20	14.8 ± 1.1 > 36	1273 ± 232 0
L. *grayi* NCTC 10815	0 21	0 20	12.2 ± 1.3 > 36	1003 ± 106 0
L. *murrayi* NCTC 10812	0 21	0 20	17.3 ± 0.5 > 36	995 ± 60 0
J. *denitrificans* NCTC 10816	0 21	0 20	19.2 ± 2.5 > 36	935 ± 23 0

(Table 4.45 continued overleaf)   (N=6)

Test species	Concentration of proflavine hydrochloride (mg dm^{-3})	moxalactam (mg dm^{-3})	DT (h) $\pm$ SD	Impedance magnitude (BUIC) $\pm$ SD
Mixed culture 1 + L. monocytogenes NCTC 11994	21	20	7.5$\pm$0.3	1371$\pm$ 7
Mixed culture 1 + L.welshimeri NCTC 11857	21	20	9.5$\pm$0.1	1125$\pm$ 23
Mixed culture 1 + L. innocua NCTC 11288	21	20	14.4$\pm$0.8	942$\pm$149
Mixed culture 1 - Listeria species	21	20	> 36	0
Mixed culture 2 + L. monocytogenes NCTC 11994	21	20	7.4$\pm$1.2	1003$\pm$ 89
Mixed culture 2 + L.welshimeri NCTC 11857	21	20	8.5$\pm$1.1	1140$\pm$ 50
Mixed culture 2 + L. innocua NCTC 11288	21	20	10.2$\pm$0.1	1257$\pm$ 3
Mixed culture 2 - Listeria species	21	20	> 36	0
Mixed culture 3 + L. monocytogenes NCTC 11994	21	20	6.3$\pm$0.1	1162$\pm$ 38
Mixed culture 3 + L.welshimeri NCTC 11857	21	20	10.9$\pm$1.0	1014$\pm$ 80
Mixed culture 3 + L. innocua NCTC 11288	21	20	11.3$\pm$0.7	1184$\pm$ 45
Mixed culture 3 - Listeria species	21	20	> 36	0

Key   (N=6)

\# = One artefactual curve produced

* = Two artefactual curves produced

Mixed Culture 1 = Lact. lactis NCIB 6681, Ent. faecalis NCIB 775, M. roseus NCIB 8175 and B.subtilis NCIB 8703.

Mixed Culture 2 = Ps. aeruginosa NCIB 950 and E. coli NCIB 86.

Mixed Culture 3 = Lact. lactis NCIB 6681, Ent. faecalis NCIB 775, M. roseus NCIB 8175 and B. subtilis NCIB 8703, Ps. aeruginosa NCIB 950 and E. coli NCIB 86.

Table 4.45   The effect of a combination of proflavine hydrochloride and moxalactam on the impedimetric responses of individual test species and mixed cultures.

L. monocytogenes NCTC 11994 and the non Listeria
components of these mixed cultures (that is, their
individual impedance DT's and magnitudes in unsupplemented
medium) are given in Table 4.44.

L. monocytogenes, L. innocua and L. welshimeri were
the only monocultures detected within 36 h in doubly
supplemented GNB. Similarly mixed cultures containing one
of these species were detected while those lacking these
species were not.

Of significance L. monocytogenes NCTC 10357 (serotype
1a), which had not been detected in GNB supplemented with
acriflavine hydrochloride and moxalactam (Table 4.40),
consistently produced detection curves in GNB supplemented
with proflavine hydrochloride and moxalactam within the
time course of the assay. All detection curves were of
good morphology and magnitude but the DT was delayed by an
average of 18.7 h compared with the respective DT in
unsupplemented medium. Similarly L. monocytogenes strains
NCTC 7973 (serotype 1a), NCTC 5348 (serotype 2) and NCTC
10527 (serotype 4b), whose impedimetric detection had been
inconsistent, being either severely delayed or totally
inhibited, in GNB supplemented with acriflavine
hydrochloride and moxalactam (Table 4.41), always produced
detection curves. Addition of proflavine hydrochloride and
moxalactam to GNB caused maximum delay in the detection of
L. monocytogenes NCTC 10527 where the DT was increased by
an average of 10.8 h compared with the respective value in
unsupplemented medium.

Using routine conditions (see 2.3, 2.6 & 2.6.1) the

impedimetric responses of a range of <u>Listeria</u> food isolates (see 2.1) were monitored for 36 h in either unsupplemented GNB or GNB supplemented with 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam. The results are tabulated in Table 4.46.

Both non haemolytic (I1 and I2) and haemolytic (I3 to I10) <u>Listeria</u> food isolates produced detection curves in the doubly supplemented medium. Addition of the selective agents caused delays in detection varying from 4.2 h (I3) to 18.1 h (I9) compared with the respective DT's in unsupplemented medium.

Additional experiments were carried out to determine the minimum concentration of <u>L. monocytogenes</u> detectable by impedimetric assay in 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam supplemented GNB. Using routine methods (see 2.3, 2.4, 2.6, 2.6.1 & 2.6.2) serially diluted samples of <u>L. monocytogenes</u> NCTC 5348 were simultaneously monitored for TVC, DT and impedance magnitude. The results are tabulated (Table 4.47) and shown graphically (Fig. 4.2). <u>L. monocytogenes</u> NCTC 5348 was chosen as the test strain in these experiments as <u>L. monocytogenes</u> NCTC 11994 had been found to exhibit uncharacteristic resistance to other combinations of antimicrobial agents compared with other members of the genus <u>Listeria</u> (see Table 4.40 & 4.41).

| Test species | Concentration of | | Impedance | |
	proflavine hydrochloride (mg dm^{-3})	moxalactam (mg dm^{-3})	DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
Food isolate I1	0	0	7.7$\pm$1.9	998$\pm$ 184
	21	20	13.7$\pm$3.9	839$\pm$ 459
Food isolate I2	0	0	9.3$\pm$0.6	834$\pm$ 19
	21	20	14.0$\pm$2.3	936$\pm$ 54
Food isolate I3	0	0	10.7$\pm$0.3	1073$\pm$ 38
	21	20	14.9$\pm$1.1	957$\pm$ 84
Food isolate I4	0	0	9.1$\pm$2.1	1025$\pm$ 49
	21	20	18.9$\pm$0.4	834$\pm$102
Food isolate I5	0	0	8.7$\pm$0.7	1074$\pm$ 58
	21	20	20.2$\pm$3.5	1023$\pm$ 68
Food isolate I6	0	0	15.1$\pm$3.1	867$\pm$ 34
	21	20	23.1$\pm$2.8	959$\pm$ 72
Food isolate I7	0	0	12.2$\pm$0.7	1236$\pm$212
	21	20	18.6$\pm$3.1	1037$\pm$184
Food isolate I8	0	0	12.4$\pm$1.8	1231$\pm$241
	21	20	25.0$\pm$4.6 #	1102$\pm$143
Food isolate I9	0	0	8.0$\pm$0.1 *	1152$\pm$ 87
	21	20	26.1$\pm$1.5	1076$\pm$ 33
Food isolate I10	0	0	11.9$\pm$1.3	1310$\pm$131
	21	20	28.0$\pm$1.1 #	1285$\pm$ 75

(N=6)

Key
= One artefactual curve produced
* = Two artefactual curves produced

Table 4.46 The effect of a combination of proflavine hydrochloride and moxalactam on the impedimetric responses of _Listeria_ food isolates.

TVC of inoculum ($CFU\ cm^{-3} \pm SD$)	Concentration of proflavine hydrochloride ($mg\ dm^{-3}$)	moxalactam ($mg\ dm^{-3}$)	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
Control				
$6.8 \pm 1.2 \times 10^{4}$	0	0	8.7 ± 0.5	1049 ± 14
	21	0	12.1 ± 0.4	1030 ± 21
$6.8 \pm 1.2 \times 10^{7}$	21	20	4.5 ± 0.2	1022 ± 187
$6.8 \pm 1.2 \times 10^{6}$	21	20	5.9 ± 0.4	952 ± 78
$6.8 \pm 1.2 \times 10^{5}$	21	20	9.7 ± 2.1	1265 ± 230
$6.8 \pm 1.2 \times 10^{4}$	21	20	13.3 ± 1.8	1176 ± 180
$6.8 \pm 1.2 \times 10^{3}$	21	20	18.3 ± 0.1	1194 ± 35
$6.8 \pm 1.2 \times 10^{2}$	21	20	20.9 ± 0.4	1054 ± 220
$6.8 \pm 1.2 \times 10^{1}$	21	20	27.1 ± 3.2	779 ± 309
$6.8 \pm 1.2 \times 10^{0}$	21	20	$29.2 \pm 4.3 (3)$ ⌀,	961 ± 113
			$> 36 (1)$ ⌀	0,

(N=4)

Key

⌀ = Number of curves of each type in brackets

Table 4.47 Impedimetric responses of a range of inoculum concentrations of **L. monocytogenes** NCTC 5348 in 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam supplemented GNB.

Electrical curves of good morphology were produced throughout the range of inoculum concentrations used and a significant negative correlation ($r = - 0.9588$, $p = 0.05$ %) was observed between inoculum concentration and DT (Fig. 4.2). **L. monocytogenes** NCTC 5348 was detected on all occasions except in one reaction well inoculated with the lowest concentration tested. In this case it can be assumed that there were no microbial cells
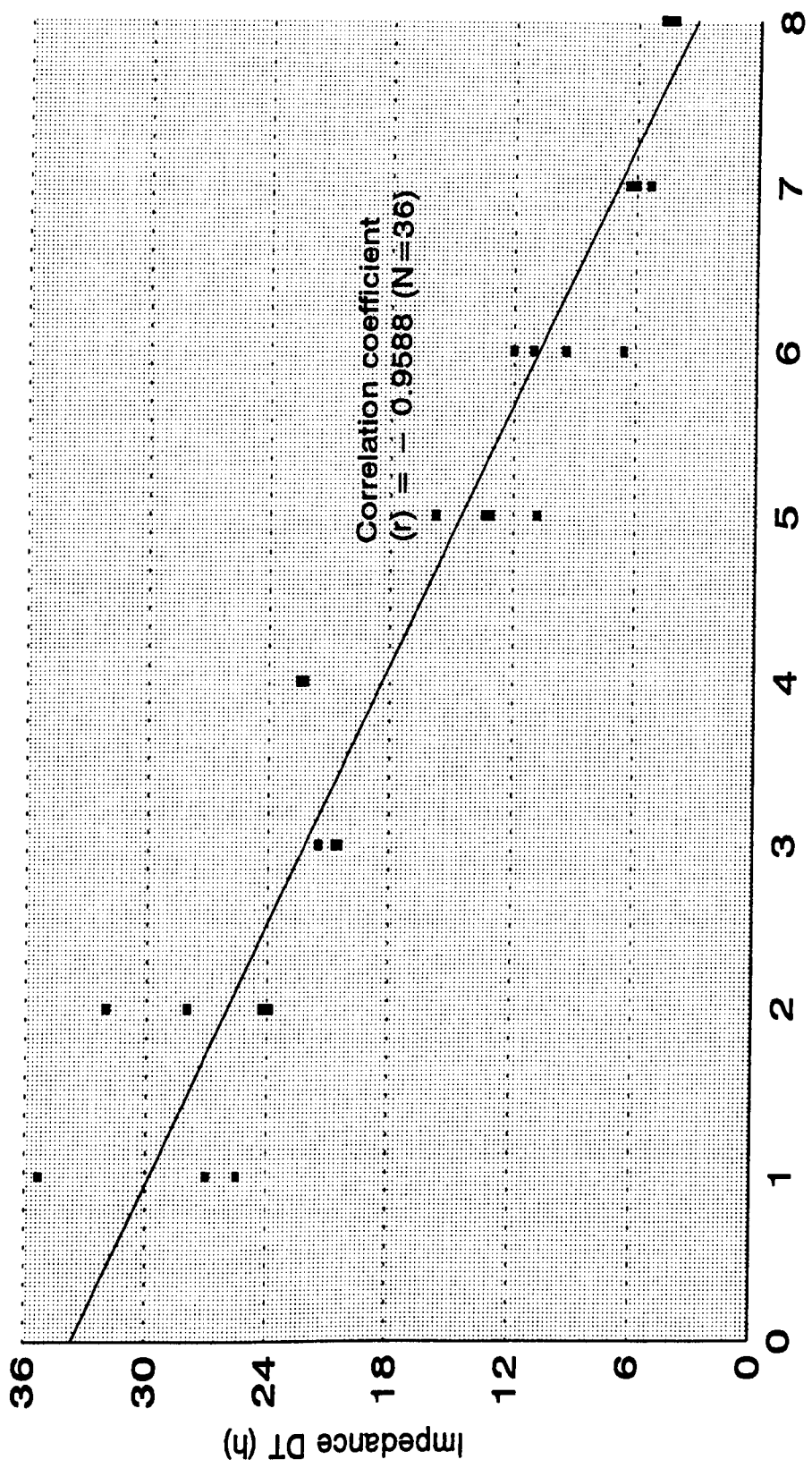
Fig. 4.2 Relationship between inoculum concentration and DT of L. monocytogenes NCTC 5348 in 21 mg dm⁻³ proflavine hydrochloride and 20 mg dm⁻³ moxalactam supplemented GNB.

present in the inoculum. The impedimetric system is therefore capable of detecting less than 10 CFU of L. monocytogenes per reaction well and hence is potentially capable of detecting a single cell.

The production of detection curves by extracellular enzymes was also investigated. However, a cell free extract of a 24 h culture of L. monocytogenes NCTC 11994 (see 2.10) did not produce detection curves in either unsupplemented GNB or GNB supplemented with 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam (data not shown).

In conclusion, GNB doubly supplemented with 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam allowed the selective impedimetric detection of strains of L. monocytogenes, L. welshimeri and L. innocua by the suppression of the metabolism of a variety of Gram positive and Gram negative species. The undesirable false positive detection of L. welshimeri and L. innocua is less important than the pathogen L. monocytogenes remaining undetected, a small number of false positive results being preferable to even a single false negative.

4.4.6 Evaluation of the effect of fungal species and antifungal agents on the impedimetric responses of L. monocytogenes.

Work was undertaken to determine the effects of the fungal species Penicillium claviforme and P. roqueforte and of the antifungal agents amphotericin B and ketoconazole on the electrical responses of L. monocytogenes.

Using routine conditions (see 2.3, 2.6 & 2.6.1) the impedimetric responses of monocultures of L. monocytogenes NCTC 11994, P. claviforme and P. roqueforte, and of a mixed culture of the three test species were monitored for 36 h in unsupplemented GNB or GNB supplemented with 21 mg dm^{-3} proflavine hydrochloride, 20 mg dm^{-3} moxalactam and 0 to 60 mg dm^{-3} of either amphotericin B or ketoconazole. As a result of preliminary experiments (see 2.4) (data not shown), 0.1 cm^3 of an undiluted 24 h malt extract broth culture was chosen as inoculum for both fungal monocultures. The inoculum of the mixed culture comprised 0.1 cm^3 of a 10^{-2} dilution of an appropriately grown 24 h culture of each of the three test species. The results are tabulated in Table 4.48.

Neither fungal species tested produced a detection curve within 36 h in unsupplemented GNB or in GNB supplemented with proflavine hydrochloride and moxalactam. However, in the presence of the antifungal agents a number of detection curves (14 out of 56) occurred at approximately 20 h. As fungi generally decrease the conductance component of the impedimetric signal, whereas bacteria increase it (Connolly et al., 1988) the production of detection curves by fungal species was not expected. Possibly these sporadic detection curves were caused by the release of intracellular fungal material, the mode of action of amphotericin B and of ketoconazole being to alter the permeability of fungal cell membranes and hence allow leakage of intracellular materials (Gilman

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/-----------------------------------------------------\
|     Controls                                        |
|-----------------------------------------------------|
|  Test          Concentration of          Impedance  |
|  species    proflavine   moxalactam    DT    magnitude |
|          hydrochloride  (mg dm^-3)   (h) ± SD  (BUIC) ± SD |
|          (mg dm^-3)                                  |
|-----------------------------------------------------|
| L.              0           0      11.0±1.7    1298± 136 |
| monocytogenes   21         20      12.8±4.0    1161± 119 |
| NCTC 11994                                           |
|-----------------------------------------------------|
| P.              0           0       > 36          0  |
| claviforme      21         20       > 36 (6) *    0  |
| ATCC 10426                                           |
|-----------------------------------------------------|
| P.              0           0       > 36          0  |
| roqueforte      21         20       > 36          0  |
| NCTC 588                                             |
|-----------------------------------------------------|
|Mixed culture    0           0       7.8±3.1    1239±186 |
|    $            21         20       9.3±4.3    1212± 93 |
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(Table 4.48 continued below)   (N=8)

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/------------------------------------------------------------------------\
|    Amphotericin B                                                      |
|------------------------------------------------------------------------|
|  Test          Concentration of                        Impedance       |
|  species  proflavine  moxalactam  amphotericin B    DT    magnitude    |
|        hydrochloride  (mg dm^-3)   (mg dm^-3)    (h) ± SD  (BUIC) ± SD  |
|        (mg dm^-3)                                                       |
|------------------------------------------------------------------------|
| L.          21        20        20      13.0±2.9       1010± 164 |
| monocytogenes 21      20        40      12.6±2.7        955± 171 |
| NCTC 11994  21        20        60      12.5±2.8 #     1016±  22 |
|------------------------------------------------------------------------|
| P.          21        20        20     > 36 (5)δ, #      0 (5) |
| claviforme                              20.9±0.4 (2)  1090± 50 (2)|
| ATCC 10426  21        20        40     > 36 #            0     |
|             21        20        60     > 36 (6) δ, #     0 (6) |
|                                         21.0 (1)       1093 (1) |
|------------------------------------------------------------------------|
| P.          21        20        20     > 36 (5) δ, *     0 (5) |
| roqueforte                              20.5 (1)       1154 (1) |
| NCTC 588    21        20        40     > 36 (4) δ, *     0 (4) |
|                                         19.3±1.2 (2)  1381±124 (2)|
|             21        20        60     > 36 #            0     |
|------------------------------------------------------------------------|
| Mixed culture 21      20        20      9.6±4.1       1117±159 |
|    $        21        20        40      9.5±4.3       1112±187 |
|             21        20        60      9.4±4.4 #     1031± 68 |
\------------------------------------------------------------------------/
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(Table 4.48 continued overleaf)   (N=8)

Ketoconazole					
Test species	proflavine hydrochloride (mg dm^{-3})	Concentration of moxalactam (mg dm^{-3})	ketoconazole (mg dm^{-3})	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
L.	21	20	20	12.8$\pm$2.6 *	1019$\pm$ 120
monocytogenes	21	20	40	14.9$\pm$3.1 #	1074$\pm$ 80
NCTC 11994	21	20	60	11.3$\pm$3.0 Ç	1030$\pm$ 132
P.	21	20	20	> 36 #	0
claviforme	21	20	40	> 36 (6) ⓪, #	0 (6)
ATCC 10426				20.5 (1)	1135 (1)
	21	20	60	> 36 (4) ⓪, #	0 (4)
				21.0$\pm$0.5 (2)	1122$\pm$ 7 (2)
P.	21	20	20	> 36 *	0
roqueforte	21	20	40	> 36 (6) ⓪,	0 (6)
NCTC 588				20.5$\pm$0.1 (2)	1135$\pm$ 8 (2)
	21	20	60	> 36 (6) ⓪,	0 (6)
				21.0$\pm$0.8 (2)	1122$\pm$ 6 (2)
Mixed culture	21	20	20	12.7$\pm$2.7 Ç	1124$\pm$ 16
$	21	20	40	9.9$\pm$4.1	1072$\pm$176
	21	20	60	11.7$\pm$2.9	1069$\pm$123

(N=8)

Key
\# = One artefactual curve produced
* = Two artefactual curves produced
Ç = Three artefactual curves produced
⓪ = Number of curves of each type in brackets
$ = Each mixed culture consisted of:
   P. claviforme ATCC 10426,
   P. roqueforte NCTC 588 and
   L. monocytogenes NCTC 11994.

Table 4.48 The effect of a combination of proflavine hydrochloride, moxalactam and amphotericin B or ketoconazole on the impedimetric responses of L. monocytogenes NCTC 11994, P. claviforme and P. roqueforte.

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et al., 1985; Sigma Technical Services, personal communication, 1993).

Even the highest concentration of amphotericin B and ketoconazole tested, 60 mg dm^{-3}, did not cause a delay in the DT of L. monocytogenes. However, whether antifungal agents were added or not, the presence of fungal species did not hinder the detection curves of the target species.

It was concluded therefore that the use of antifungal agents in the impedimetric system would not be necessary as the fungal species were incapable of producing detection curves within 36 h in GNB supplemented with 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam.

4.4.7 Evaluation of the effect of aesculin and ammonium-ferric citrate on the impedimetric responses of test species.

Aesculin hydrolysis has been used in traditional methodology for the detection of Listeria spp. In the presence of iron all Listeria spp. hydrolyse aesculin causing blackening around their colonies on plating media (Dominguez et al., 1984; Curtis et al., 1989a; bioMérieux, 1992b).

The aim of this work was to determine the effectiveness of incorporating aesculin hydrolysis as a secondary colorimetric detection system in an impedimetric assay for L. monocytogenes. Using routine conditions (see 2.3, 2.6 and 2.6.1) the impedimetric responses of L. monocytogenes NCTC 11994 were monitored for 36 h in

unsupplemented GNB, unsupplemented ANB (see 2.2 & 2.9), and ANB supplemented with 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam. In addition the colour of the reaction wells was observed every 4 h. The results are recorded in Table 4.49.

The DT of L. monocytogenes in unsupplemented ANB was 1.5 h shorter than in unsupplemented GNB. This finding was unexpected as aesculin has been reported to be not as readily usable as a growth enhancing substrate as glucose (Lachia, 1990). The corresponding DT in the doubly supplemented ANB was not unduly delayed. Furthermore all electrical curves were of good morphology and magnitude, and significantly there was an obvious and distinct blackening in the reaction wells after 24 h. Therefore the colorimetric test could be used in conjunction with the impedimetric assay for L. monocytogenes.

Impedimetric medium	Concentration of proflavine hydrochloride (mg dm^{-3})	moxalactam (mg dm^{-3})	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
GNB	0	0	11.1$\pm$0.1	951$\pm$ 65
ANB	0	0	9.6$\pm$0.2	1212$\pm$ 48
ANB	21	20	12.0$\pm$0.4	962$\pm$ 89

(N=4)

Table 4.49 The effect of aesculin and ammonium-ferric citrate on the impedimetric responses L. monocytogenes NCTC 11994.

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4.4.8 Evaluation of the effect of L-rhamnose on the impedimetric responses of test species.

Strains of L. welshimeri and L. innocua have been shown to exhibit variable growth responses on the sugar L-rhamnose whereas all L. monocytogenes strains are capable of utilising this sugar (McLauchlin, 1987; Notermans et al., 1989b). In an attempt to prevent the detection of L. welshimeri and L. innocua the impedimetric responses of these species were therefore investigated in nutrient broth enriched with L-rhamnose rather than L-glucose.

Using routine conditions (see 2.3, 2.6 and 2.6.1), the impedimetric responses of the test species were monitored for 36 h in unsupplemented GNB and RNB (see 2.2) as well as in both types of media supplemented with 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam. Cultures previously grown in GNB and in RNB were used as inocula. As in the latter case preliminary experiments (data not shown) had indicated that growth was poor after 24 h, 10^{-2} dilutions were used rather than the normal 10^{-4} dilutions. The results are tabulated in Table 4.50.

Whilst neither GNB nor RNB grown L. welshimeri produced detection curves in RNB, this species produced detection curves in both unsupplemented GNB and doubly supplemented GNB on all occasions. This indicates that the enzymes necessary for L-rhamnose utilisation are neither synthesised constitutively nor inducibly in the strain tested.

Test species (and initial growth medium)	Impedimetric medium	Concentration of proflavine hydrochloride (mg dm^{-3})	moxalactam (mg dm^{-3})	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
L. welshimeri NCTC 11957 (GNB)	GNB	0	0	12.5$\pm$0.4	941$\pm$ 10
		21	20	20.1$\pm$2.0	1052$\pm$ 124
	RNB	0	0	> 36	0
		21	20	> 36	0
L. welshimeri NCTC 11957 (RNB)	GNB	0	0	8.8$\pm$0.4	822$\pm$ 16
		21	20	25.8$\pm$0.9 #	803$\pm$ 39
	RNB	0	0	> 36	0
		21	20	> 36	0
L. innocua NCTC 10889 (GNB)	GNB	0	0	20.7$\pm$0.1	455$\pm$ 10
		21	20	> 36	0
	RNB	0	0	> 36	0
		21	20	> 36	0
L. innocua NCTC 10889 (RNB)	GNB	0	0	15.7$\pm$0.4	497$\pm$ 11
		21	20	> 36	0
	RNB	0	0	> 36 #	0
		21	20	> 36	0
L. innocua NCTC 11288 (GNB)	GNB	0	0	14.2$\pm$0.2	1013$\pm$ 3
		21	20	28.2$\pm$1.5	924$\pm$ 174
	RNB	0	0	19.4$\pm$2.2	443$\pm$ 6
		21	20	32.5$\pm$0.5	253$\pm$ 82
L. innocua NCTC 11288 (RNB)	GNB	0	0	11.9$\pm$0.7 *	1083$\pm$ 87
		21	20	23.6$\pm$1.3 #	1249$\pm$ 28
	RNB	0	0	11.6$\pm$0.6	618$\pm$ 6
		21	20	29.8$\pm$0.8	591$\pm$ 13

(N=4)

Key
= One artefactual curve produced
* = Two artefactual curves produced

Table 4.50 The effect of L-rhamnose on the impedimetric responses of **L. welshimeri** and **L. innocua**.

Both GNB and RNB grown <u>L. innocua</u> NCTC 10889 produced late detection curves in unsupplemented GNB but not in the other media. This late detection was most likely due to a low concentration of cells in the inoculum added to these wells. The effects of the substitution of L-rhamnose for glucose therefore could not be determined for this strain. However GNB and RNB grown <u>L. innocua</u> NCTC 11288 produced detection curves in both unsupplemented and doubly supplemented GNB and RNB. The use of L-rhamnose rather than glucose therefore did not prevent the detection of this test strain.

The variable results obtained in this work were as expected, L-rhamnose preventing the detection of one test species but not affecting another. As L-rhamnose inhibited the impedimetric detection of <u>L. welshimeri</u>, its incorporation into the impedimetric medium was evaluated further.

Using routine conditions (see 2.3, 2.6 & 2.6.1) the impedimetric responses of a range of strains of <u>L. monocytogenes</u> and <u>Listeria</u> food isolates were monitored for 36 h in unsupplemented RNB and RNB supplemented with 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam. The results are given in Table 4.51.

Non-haemolytic and haemolytic <u>Listeria</u> food isolates were detected on all occasions in the doubly supplemented RNB. However, all strains of <u>L. monocytogenes</u> except NCTC 11994 remained undetected. This finding precluded the further use of L-rhamnose in an impedimetric assay for <u>L. monocytogenes</u>.

Test species	Concentration of proflavine hydrochloride $(mg\ dm^{-3})$	moxalactam $(mg\ dm^{-3})$	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
L. monocytogenes NCTC 7973 (serotype 1a)	0 21	0 20	13.3$\pm$0.2 > 36 #	647$\pm$ 7 0
L. monocytogenes NCTC 10357 (serotype 1a)	0 21	0 20	13.1$\pm$0.7 > 36	185$\pm$ 63 0
L. monocytogenes NCTC 5348 (serotype 2)	0 21	0 20	13.6$\pm$0.2 > 36 (3)Ϭ, 34.0 (1)Ϭ	531$\pm$ 107 0, 434
L. monocytogenes NCTC 10527 (serotype 4b)	0 21	0 20	14.6$\pm$0.3 > 36	253$\pm$ 15 0
L. monocytogenes NCTC 11994 (serotype 4b)	0 21	0 20	12.7$\pm$0.1 14.4$\pm$0.2	517$\pm$ 20 886$\pm$ 27
Listeria food isolate I2	0 21	0 20	8.8$\pm$0.5 9.7$\pm$0.9	895$\pm$ 9 822$\pm$ 90
Listeria food isolate I3	0 21	0 20	11.1$\pm$0.8 14.8$\pm$0.7	737$\pm$ 53 547$\pm$ 23
Listeria food isolate I4	0 21	0 20	14.5$\pm$0.7 # 23.8$\pm$3.5	774$\pm$ 33 642$\pm$ 78

(N=4)

Key

\# = One artefactual curve produced

Ϭ = Number of curves of each type in brackets

Table 4.51 The effect of L-rhamnose on the impedimetric responses of strains of L. monocytogenes and Listeria food isolates.

4.5 Discussion

The results reported in this chapter generally correspond well with those obtained by conventional methods of testing antimicrobial activity. For example, the range of concentrations of nalidixic acid reported to be selective for L. monocytogenes (Kramer & Jones, 1969; Gilman et al., 1985; Doyle & Schoeni, 1986; Buchanan et al., 1987; Farber et al., 1987, 1988), was found to inhibit the growth and electrical responses of E. coli (Tables 4.1 & 4.2). Similarly the concentrations of potassium tellurite found to be effective against Gram negative organisms by Schoer (1944) and Gray et al. (1950) was confirmed by impedimetric assay (Tables 4.14 & 4.15). However, there were some discrepancies. For example moxalactam had been found to be ineffective against Pseudomonas spp. (Yogev, 1986), yet this agent inhibited the production of detection curves by Ps. fluorescens (Table 4.8). Moxalactam may have a lower inhibitory affect on Ps. fluorescens than on some other test species, just preventing this organism reaching the threshold level required for detection by impedance microbiology. Nevertheless, the high degree of correlation between antibiotic sensitivity as measured by minimum inhibitory concentration and by impedimetric methods found by others (Hogg et al., 1987) can be confirmed.

It has been reported that some selective agents can affect the quality of electrical curves (Adak et al., 1987a; Arnott et al., 1988). This was encountered in the

current work. For example, nalidixic acid was found to cause a delay in the DT and decrease the magnitude of the impedimetric responses of test species (see 4.2.1). Agents capable of improving the morphology of electrical curves were also recognised. For example, lithium chloride was found to enhance the magnitude of curves (Table 4.12). It could be argued that this effect is due to the additional electrolytes in the medium (viz. the lithium and chloride ions themselves), impedimetric signals being strongly dependent on salt concentration (Richards et al., 1978). However potassium tellurite supplemented GNB yielded low magnitude impedance curves (see 4.2.6). As all concentrations of lithium chloride examined increased impedance magnitude and potassium tellurite consistently decreased this parameter, the effects of these agents on electrical responses are not likely to be due to the amount of salt (w/v) in solution. As both metal ions carry the same charge, the electrochemical effect may be related to the size of the ions in solution. The smaller lithium ion being more mobile would therefore be expected to be more active electrically. However, due to hydration it is possible that these metal ions are the same size, and therefore the differential modification of electrical responses by lithium chloride and potassium tellurite may be due to the comparative effects of the chloride and tellurite ion.

It was noticed that some mixed cultures influenced the impedimetric responses of L. monocytogenes. Lact. lactis reduced the magnitude of impedance curves

produced by the target organism in thallous acetate supplemented GNB (Table 4.5), while in acriflavine hydrochloride supplemented GNB a broad spectrum of eight microorganisms (including Ps. fluorescens, a microorganism resistant to the selective agent being evaluated) did not have any effect on their morphology (Table 4.20). The variable magnitude of impedance curves produced by L. monocytogenes in acriflavine hydrochloride and moxalactam supplemented GNB in the presence of different mixed cultures (Table 4.36) would preclude detection by a qualitative impedimetric method. Under these conditions impedimetric detection of L. monocytogenes could therefore only be achieved by a quantitative type assay.

Fluctuations in impedance magnitudes were found between batches of medium (see Table 4.39). This has also been reported by others (Bolton, 1990). Such variation could be caused by different extents of medium caramelisation affecting the mobility of ions. This effect might be eliminated by autoclaving for sufficient time to ensure that all heat catalysed reactions have occurred, or by filter sterilising individual medium components and combining them aseptically. However, batch to batch fluctuations would not affect a quantitative type assay.

Reproducibility of curve criteria is important in either type of impedimetric assay. The number of artefactual curves which were produced in even the most highly controlled conditions in this work dictates that any electrical assay must be performed in at least duplicate. The occurrence of false positive results needs

to be low enough to minimise the need for confirmatory tests, and more seriously, in an industrial situation false negative results would have to be avoided to prevent the release of contaminated foods.

Of the range of selective agents tested, proflavine hydrochloride and moxalactam met the necessary criteria for use in the impedimetric assay for L. monocytogenes. Both agents have had previous medical applications. Aminoacridines have been used to irrigate deep infected wounds as their antibacterial action takes place at high dilution and at one time they were thought not to harm mammalian tissue (Poate, 1944). Proflavine hydrochloride is less toxic than acriflavine hydrochloride (Albert, 1944). Moxalactam has been used to treat infections in many areas except those in the US where L. monocytogenes is prevalent (Yogev, 1986). A combination of these two agents was found to inhibit the impedimetric responses of all species tested apart from L. monocytogenes, L. innocua and L. welshimeri (see 4.4.5).

In sensitive cells, the flat proflavine hydrochloride molecules become intercalated into DNA between base pairs. They are bound by Van der Waals forces and by ionic bonds to the phosphate ions of the DNA backbone (Lerman, 1961). Polymerases are then prevented from synthesising bacterial DNA and RNA (Hurwitz et al., 1962). Bacterial DNA is more vulnerable to toxic agents than the DNA of higher forms of life because of its lack of protection by a either a nuclear membrane or histone molecules (Albert, 1985). Once they have penetrated the plasma membrane the aminoacridine

molecules therefore have unlimited access to their target site. The bacteriostatic action of aminoacridines has been found to be proportional to the degree of ionisation (Albert, 1944). Ionisation is required for the toxic agent to remain bound, as neutral aminoacridines easily leave the receptor exchanging with the sodium and potassium ions that bathe the area. Although ions require an energy consuming mechanism in order to penetrate membranes, neutral aminoacridines can pass through the plasma membrane and reionise in the aqueous cytoplasm (proflavine hydrochloride is 99 % ionised at a pH of 7.3 at 37 °C) (Albert, 1965; 1985).

ß lactam antibiotics such as moxalactam affect the transpeptidase enzyme which catalyses the peptide crosslinks of the peptidoglycan cell wall (Mandell & Sande, 1991). In addition a number of so called penicillin binding proteins act as receptors for these antibiotics. E. coli, for example, has at least seven and include proteins necessary for maintenance of the rod like shape and for septum formation. Penicillin binding proteins vary in their affinity for different ß lactams (Mandell & Sande, 1991). After treatment with ß-lactams susceptible growing bacteria rupture under their own osmotic pressure (Albert, 1985).

For antimicrobial agents to exert their action they must have access to their relevant target sites. The cytological and biochemical differences between the various types of microbial cell are the basis of the selective activity of toxic agents and dictate whether

these agents can reach the receptor, or are degraded, deactivated or by storage prevented from contact with the target sites.

Major differences between microorganisms occur in their cell walls. The outer surfaces of some bacteria are coated by capsules which are capable of affecting the uptake of ionised molecules into the cell, promoting entry of cations and retarding anions. Encapsulated Ps. aeruginosa variants are more resistant than non capsulated cells to anionic penicillins (Chopra & Ball, 1982). Fiedler (1988) found no evidence for the presence of a capsule in Listeria, an observation which corresponds with the resistance of this genus to the cationic acridine dyes (see 4.2.8 & 4.2.9).

The walls of Gram positive bacteria are 15 to 30 nm thick and composed mainly of peptidoglycan, a polymer of β 1,4 linked amino sugars cross-linked by short peptides. While the sugar residues are almost always N-acetyl glucosamine and N-acetyl muramic acid, the composition of the peptides and mode of cross linkage vary between bacteria (Schleifer and Kandler, 1972; Chopra & Ball, 1982; Albert, 1985). In many Gram positive organisms other polymers, such as teichoic acids and teichuronic acids, are covalently linked to the amino sugar chains, the quantity of these varying with growth conditions (Chopra & Ball, 1982). The cell wall of members of the genus Listeria has been found to be typical of Gram positive organisms (Fiedler, 1988; Jones, 1989; Alvarez-Dominguez et al., 1993). Their peptidoglycan

contains a dipeptide N-acetylmuramyl D-alanyl
L-isoglutamine which is directly cross linked by
mesodiaminopimelic acid and is associated with teichoic
acids, lipoteichoic acids and long chain fatty acids.
L-rhamnose is also present (Ralovich, 1984;
Alvarez-Dominguez et al., 1993). It is possible that the
resistance of L. monocytogenes to some selective agents
could be due to the presence of charged cell wall
components affecting their accumulation at the cell wall
and thus reducing access to target sites. Cationic
acridine dyes and anionic ß lactam antibiotics would be
repelled by similarly charged components and attracted by
oppositely charged ions. Similarly, the lipophilic
component of lipoteichoic acids in the cell walls could
repel hydrophilic antimicrobial agents such as proflavine
hydrochloride.

The walls of Gram negative bacteria are more
complicated. The peptidoglycan layer, constituting 5 to
20 % of the cell wall mass, is firmly attached on the
inner side to the plasma membrane while its outer side is
covered with another membrane, consisting of a lipid
bilayer with lipoprotein and lipopolysaccharide (Mandell &
Sande, 1991). This outer membrane of Gram negative
bacteria is of primary importance in excluding hydrophilic
materials and accounts for the resistance of these cells
to some ß lactam antibiotics, which must traverse it to
reach their receptor sites (Gilbert, 1984). The
hydrophobic outer membrane could be the reason for the
resistance of Pseudomonas spp. to acridine dyes (see 4.2.8

& 4.2.9). However, no evidence of an outer membrane has been found in Gram positive L. monocytogenes (Fiedler, 1988), and therefore this morphological feature cannot be responsible for resistance of the target species to either moxalactam or proflavine hydrochloride (see 4.2.3 & 4.2.9).

Some outer membrane proteins are involved in facilitated diffusion of specific solutes (including antibiotics) across the membrane (Chopra & Ball, 1982), while others, such as porins, allow relatively non specific passive diffusion of hydrophilic low molecular weight solutes including most cephalosporin antibiotics (Albert, 1985; Mandell & Sande, 1991). The inhibition of the impedimetric responses of Ps. fluorescens and E. coli by moxalactam (see 4.2.3) could therefore be accounted for by the antibiotic gaining access to the penicillin binding proteins via these uptake systems.

Fungal cell walls consist of a mosaic of carbohydrates with some lipids and proteins. The predominant carbohydrate in filamentous fungi is chitin, a polymeric N-acetyl glucosamine (Albert, 1985). However, as the Penicillium species tested in this work were sensitive to proflavine and moxalactam it was not necessary to exploit the cytological differences between these eukaryotic cells and the prokaryotic target organism L. monocytogenes in the development of the impedimetric assay.

The comparative distribution of toxic agents relies mainly upon their ability to pass through membranes

(Albert, 1985). The Gram positive cytoplasmic membrane (or plasma membrane) and the inner membrane of Gram negative microorganisms are similar. Both are approximately 6 to 10 nm thick, sometimes extending into the cytoplasm as simple protrusions, and contain enzyme systems for electron transfer, transport of solutes, lipid synthesis and production of cell wall and envelope polymers. Their structural framework is a phospholipid bilayer which in Gram positive species is the main permeability barrier. The presence of specific facilitated diffusion and active transport carriers in bacterial membranes dictates which toxic agents are taken up (Chopra & Ball, 1982). The resistance of L. monocytogenes to aminoacridines (see 4.2.8 & 4.2.9) could therefore be due to the lack of a relevant transport system and hence the inability of these agents to gain access to their target site, the DNA. Furthermore, membrane lipids have been shown to store neutral toxic agents and membrane bound RNA cationic toxic agents (Albert, 1985). As the plasma membrane of L. monocytogenes contains 15 to 20 % neutral lipid and 1.5 % RNA (Ralovich, 1984), these components could also be implicated in the resistance of the target organism to proflavine hydrochloride (see 4.2.9).

As the receptors for cephalosporins are incorporated into the plasma membrane it is not necessary for moxalactam to penetrate this membrane. The sensitivity of microorganisms to ß lactams is thought to be related to the extent of binding to suitable receptors. For example, Vicente et al. (1990) suggested that the susceptibility

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pattern of L. monocytogenes, which is sensitive to penicillins but resistant to cephalosporins, reflects the presence of binding proteins for the former type of antimicrobial agent only.

A number of general mechanisms exist by which bacteria become resistant to toxic agents. Degradative enzymes are used by many species to increase their resistance to agents. Chopra and Ball (1982) reported that several plasmid encoded antibiotic inactivating enzymes are located in the periplasmic space between the inner and outer membranes of Gram negative bacteria, while Gram positive bacteria are known to secrete large amounts of ß lactamase (Mandell & Sande, 1991). Barbuti et al. (1992) tested 26 antimicrobial agents on 64 strains of L. monocytogenes and 102 strains of L. innocua. These authors found strains of L. monocytogenes with non plasmid mediated resistance to four antibiotics used therapeutically, viz. tetracycline, co-trimoxazole, erythromycin and clindamycin, but no evidence of plasmid borne resistance in this species. This suggests that the resistance to moxalactam exhibited by the target species (see 4.2.3) is unlikely to be plasmid mediated. Mutation can also decrease the sensitivity of cells to antibiotics, the increased use of antibiotics promoting the selection of resistant strains.

The colour change caused by aesculin hydrolysis has been used for many years in traditional plating media to indicate the presence of the pathogen L. monocytogenes (see for example Dominguez et al., 1984). This phenomenon

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was also successfully utilised by Phillips & Griffiths
(1989) in a qualitative impedimetric assay for
L. monocytogenes. In this chapter it was evaluated for
inclusion in the quantitative assay developed in current
work (see 4.4.7). The shorter DT's observed for
L. monocytogenes in ANB compared with GNB may be explained
on the basis of the availability of extra nutrients. Iron
present in the former medium only (see 2.2) has been
observed to enhance the growth of Listeria spp. (Cowart &
Foster, 1985), while glucose is liberated in the
hydrolysis of aesculin by ß-glucosidase (bioMérieux,
1992b). However, as the target organism was detected
within 13 h by impedance and only after 24 h by aesculin
hydrolysis, waiting for this characteristic to develop
would increase the assay time. In addition, as all
Listeria spp. hydrolyse aesculin, it could not be used to
differentiate L. welshimeri or L. innocua from
L. monocytogenes, and therefore would not improve
selectivity. Furthermore, as Enterococcus spp., which are
used as probiotic feed organisms for farm animals (Fuller,
1989), also cause aesculin hydrolysis (bioMérieux, 1992c),
their presence in food products could be expected to
produce false positive results in the colorimetric assay.

As both L. innocua and L. welshimeri show variable
growth responses to L-rhamnose (Rocourt et al., 1983), an
attempt was made to improve the selectivity of the
impedimetric assay by substituting the former sugar for
glucose. Unfortunately, however, a number of strains of
L. monocytogenes were not detected in this modified medium

(see 4.4.8). It has been reported that L-rhamnose, a constituent of peptidoglycan in the target species, binds complement factors which promote the uptake of L. monocytogenes by macrophages during an early stage in pathogenesis (Ralovich, 1984; Benedict, 1990; Alvarez-Dominguez et al., 1993). As metabolism of L-rhamnose by L. monocytogenes generates highly conductive lactic acid molecules (Benedict, 1990), the incorporation of this sugar into the cell wall would decrease the yield of ionic products, and may account for the lack of detection of some strains. Reduction of the impedimetric responses in RNB compared with GNB might therefore be used as an indicator of virulence.

The impedimetric assay developed for L. monocytogenes was next evaluated in foodstuffs (Chapter 5).

CHAPTER 5

EVALUATION OF THE IMPEDIMETRIC ASSAY FOR THE DETECTION AND ENUMERATION OF FOODBORNE *L. monocytogenes*

5.1 Introduction

The ultimate objective of this research is the impedimetric detection of *L. monocytogenes* from food substrates. The constituents of foods are complex, the chemical matrix and its intrinsic microflora being very variable from food to food. Food substrates therefore require careful evaluation as potentially they can influence both the selective toxicity of an impedimetric medium and the morphology of the electrical curves obtained.

Experiments using traditional detection procedures have shown that in general foods with substantial loads of contaminating microorganisms require highly selective media in order to isolate the target species, while those with low loads require less selectivity. Furthermore, antimicrobial agents suppressing non target species from one type of food do not necessarily suppress such organisms from another (Brackett & Beuchat, 1989). The physical characteristics of a food, for example particle size and pH, can also influence the recovery of target organisms (Brackett & Beuchat, 1989).

Firstenberg-Eden (1983) highlighted the importance of sample preparation in impedimetric detection of

microorganisms. She observed that the diluents in which foods were stomached affected both DT's and the quality of the electrical curves. The storage of diluted samples at either mesophilic or refrigeration temperatures prior to impedimetric testing was found to have an adverse effect on curve production, while a poor correlation between inoculum concentration and DT resulted in samples which had previously been frozen.

The suitability of a detection method is influenced by the purpose of the analysis (Brackett & Beuchat, 1989). The objectives of microbiological examinations vary; sometimes a gross count is required to determine the shelf life of a food product (Collins-Thompson & Allen, 1986), while on other occasions the specific detection of particular target organisms is necessary, as in this work. As the infectious dosage of L. monocytogenes is still unknown (WHO Working Group, 1988; Al-Ghazali & Al-Azawi, 1990; Johnson et al., 1990; Lund, 1990), a zero tolerance limit has been set and one cell of L. monocytogenes in 25 g of foodstuff is now unacceptable (Wehr, 1987). Because of the constraints imposed by the necessity of homogenising solid foods in diluents and by the volume of inocula which can be added to plates, direct plating on selective media cannot consistently detect less than 10^3 cells g^{-1} (Golden et al., 1988a; Yousef et al., 1988). Such direct techniques therefore lack sufficient sensitivity for zero tolerance assurance. While enrichment may be used to increase the concentration of the target species enough to be detected by traditional plating

methods, this has the disadvantage of precluding accurate enumeration. Enrichment prior to impedimetric analysis would also be at the cost of rapidity. However, this disadvantage may be outweighed if enrichment permitted the use of reduced levels of food substrates in the inoculum and hence lessened the risk of these interfering with the production of good quality impedance curves.

It is important that a selective system is able to detect stressed organisms as food processing can cause many sublethal injuries. Microorganisms damaged by procedures such as heat treatment, refrigeration, freeze drying, freezing and thawing, irradiation, changes in nutrition, and addition of preservatives, may retain their virulence (Lovett, 1988; Mossel, 1989). Sublethally injured cells can repair in foods stored after processing (Warburton et al., 1992), or in hospitable parts of the body after consumption resulting in disease (Mossel & van Netten, 1984). On the other hand, the additional stress caused to sublethally injured cells by the chemicals in some selective media can result in failure to detect the target organisms (Mossel, 1989).

The aim of the work described in this chapter was to evaluate different methods of sample preparation and to determine whether the impedimetric assay developed in Chapter 4 could be used to detect both unstressed foodborne L. monocytogenes and cells stressed as a result of temperature shocks. Initially a one stage direct assay was used to monitor L. monocytogenes in cheese. Later this procedure was modified and a number of enrichment regimens

were examined in an attempt to increase the sensitivity of the assay to enable zero tolerance specifications for L. monocytogenes to be adhered to.

5.2 Results

5.2.1 Comparison of the efficiency of methods of dissociating microorganisms from solid food products.

As the major food products likely to harbour the target organism, L. monocytogenes, are solid, for example cheese and paté, the first stage in any detection procedure must involve the recovery of the organisms. Three standard methods of dissociating the normal microflora from food substrates were compared, viz:

1) Homogenisation in a blender (Braun).

2) Grinding and pounding in a pestle and mortar.

3) Stomaching in a stomacher (Colworth).

In each case 100 cm^3 of 10 % (w/v) cheddar cheese in 1/4 strength Ringers solution was treated for differing time intervals and then the TVC and impedimetric responses of 0.1 cm^3 of each sample of dissociated cheese in 1.0 cm^3 GNB were determined (see 2.4, 2.6 & 2.6.1). The results are tabulated in Table 5.1.

As the same cheese was used throughout this set of experiments, if each method was equally effective then a similar colony count and corresponding DT would have been expected in all cases. From Table 5.1 it can be seen that

Dissociation method	time (minutes)	TVC (CFU g^{-1} $\pm$ SD)	DT (h) $\pm$ SD	Impedance magnitude (BUIC) $\pm$ SD
Blender	0.16	$2.5\pm0.15 \times 10^8$	11.5 ± 0.1	1063 ± 10
"	0.30	$2.6\pm0.07 \times 10^8$	11.1 ± 0.2	1038 ± 11
"	0.50	$2.8\pm0.35 \times 10^8$	11.4 ± 0.2	1212 ± 25
"	0.62	$2.5\pm0.20 \times 10^8$	11.3 ± 0.0	1255 ± 17
Pestle and mortar	0.50	$2.7\pm0.19 \times 10^8$	9.9 ± 0.0	803 ± 28
mortar	1.00	$4.2\pm0.32 \times 10^8$	9.8 ± 0.2	767 ± 7
"	1.50	$3.5\pm0.20 \times 10^8$	10.5 ± 0.3	786 ± 10
"	2.00	$4.1\pm0.27 \times 10^8$	10.3 ± 0.3	851 ± 19
Stomacher	0.50	$3.7\pm0.28 \times 10^8$	10.1 ± 0.0	791 ± 12
"	1.00	$6.0\pm0.46 \times 10^8$	10.5 ± 0.0	829 ± 50
"	1.50	$4.3\pm0.34 \times 10^8$	10.6 ± 0.0	822 ± 30
"	2.00	$6.1\pm0.49 \times 10^8$	10.5 ± 0.1	741 ± 13

(N=2)

Table 5.1 Comparison of the cell count and impedimetric responses obtained after three different methods of dissociating the normal microflora from cheddar cheese.

this did not occur. Of the three procedures investigated the stomacher separated the microorganisms most efficiently; the DT's obtained were equivalent to those for the pestle and mortar, while the TVC's were higher. Although the data were somewhat variable, dissociation appeared to be complete after 1.0 minute. Use of the blender, on the other hand, consistently resulted in greater impedance magnitudes than the other methods but it

liberated fewer microorganisms and yielded longer DT's. As DT's are not affected by the degree to which the organisms are dissociated from each other, this indicates that homogenisation in the blender caused a reduction in cell viability. The stomacher was therefore chosen to dissociate microorganisms from the solid food products used in this work.

5.2.2 Evaluation of the effect of temperature stress on the impedimetric responses of L. monocytogenes NCTC 11994.

As food processing procedures can cause sublethal injuries to cells, an investigation was carried out to determine whether the impedimetric assay could detect stressed cells of L. monocytogenes. L. monocytogenes NCTC 11994 was treated with three different temperature regimens. A 24 h GNB culture was diluted to 10^{-4} in 1/4 strength Ringers solution. 10 cm^3 volumes of the diluted culture were incubated for 1 h at 4°, 30° and 55°C. The latter temperature chosen as previous workers had found it effective in causing sublethal stress to L. monocytogenes (Warburton et al., 1992). After the incubation period, 0.1 cm^3 aliquots were transferred to Bactometer wells containing 1.0 cm^3 of either unsupplemented GNB or GNB supplemented with 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam and, using routine conditions (see 2.6 & 2.6.1), the impedimetric responses of the three sets of temperature conditioned cells were monitored for 36 h. The results are tabulated in Table 5.2.

Little difference was observed between the impedimetric responses of the control cells (viz those pre-incubated at 30 °C) and the cold stressed cells. However the DT of the heat stressed cells was increased by 4 h in comparison with the control cells in both the unsupplemented and the doubly supplemented GNB. In addition these cells produced much lower impedance magnitudes than the control and cold stressed cells. Such observations would be expected if the heat treatment had reduced the viable cell number and/or caused sublethal injuries.

It was therefore concluded that the impedimetric assay could detect temperature stressed cells. The increase in DT observed with heat stressed cells was not thought to be of sufficient duration to warrant a period of non selective enrichment.

Temperature treatment	Concentration of proflavine hydrochloride ($mg\ dm^{-3}$)	moxalactam ($mg\ dm^{-3}$)	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
30 °C for 1 h (control)	0	0	10.4$\pm$0.0	1230$\pm$ 18
	21	20	11.3$\pm$0.1	1026$\pm$ 41
4 °C for 1 h	0	0	10.9$\pm$0.1	1178$\pm$ 2
	21	20	10.9$\pm$0.0	1123$\pm$ 25
55 °C for 1 h	0	0	14.7$\pm$0.1	469$\pm$ 25
	21	20	15.4$\pm$0.2	514$\pm$ 10

(N=4)

Table 5.2 The effect of temperature stress on the impedimetric responses of L. monocytogenes NCTC 11994.

5.2.3 Evaluation of the direct impedimetric assay for the detection of L. monocytogenes in spiked cheeses.

Work was undertaken to determine whether L. monocytogenes could be detected in spiked cheeses by the impedimetric assay developed in chapter 4.

Using routine conditions (see 2.6, 2.6.1 & 2.7) the impedimetric responses of 10 % (w/v) suspensions of a variety of cheeses spiked with L. monocytogenes NCTC 11994 (at a concentration of 1×10^4 and 1×10^3 CFU g^{-1}) were monitored for 36 h in GNB supplemented with 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam. Additionally, to investigate the presence of naturally occurring L. monocytogenes in these cheeses, the impedimetric responses of unspiked samples were similarly monitored in unsupplemented and doubly supplemented GNB and conventional selective plating was undertaken (see 2.4). The impedimetric results are recorded in Table 5.3.

No evidence was obtained from either the selective plates (data not shown) or the impedimetric assay (Table 5.3) for the natural contamination of any of the cheeses with L. monocytogenes. However the target organism was detected on all occasions in the spiked cheeses by the impedimetric assay, indicating that this procedure can reveal 1×10^3 CFU L. monocytogenes g^{-1} cheese (Table 5.3). Although the different cheeses were found to affect the magnitude and shape of the electrical curves produced, all curves were of adequate morphology to enable the detection of the target species.

| Cheese | TVC of added _L. monocytogenes_ (CFU g^{-1} cheese) | Concentration of | | Impedance | |
		proflavine hydrochloride (mg dm^{-3})	moxalactam (mg dm^{-3})	DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
Edam	Unspiked	0	0	15.2$\pm$0.3	512$\pm$152
	Unspiked	21	20	> 36	0
	1 x 10^4	21	20	18.2$\pm$1.7	1438$\pm$ 17
	1 x 10^3	21	20	20.9$\pm$3.5	1405$\pm$ 81
Cheddar	Unspiked	0	0	7.9$\pm$0.3	674$\pm$ 71
	Unspiked	21	20	> 36	0
	1 x 10^4	21	20	22.9$\pm$2.9	912$\pm$ 77
	1 x 10^3	21	20	25.1$\pm$2.2	877$\pm$ 55
Gouda	Unspiked	0	0	10.0$\pm$0.4	713$\pm$ 3
	Unspiked	21	20	> 36	0
	1 x 10^4	21	20	21.1$\pm$1.7	1079$\pm$ 71
	1 x 10^3	21	20	23.3$\pm$2.5	1154$\pm$ 62
Danish blue	Unspiked	0	0	12.4$\pm$1.4	490$\pm$162
	Unspiked	21	20	> 36	0
	1 x 10^4	21	20	13.6$\pm$2.9	1354$\pm$ 6
	1 x 10^3	21	20	14.6$\pm$1.5	1365$\pm$ 59
Stilton	Unspiked	0	0	7.5$\pm$0.6	529$\pm$ 5
	Unspiked	21	20	> 36	0
	1 x 10^4	21	20	23.0$\pm$1.8	776$\pm$ 10
	1 x 10^3	21	20	26.4$\pm$0.3	782$\pm$ 44
Brie	Unspiked	0	0	3.1$\pm$0.2	1367$\pm$ 5
	Unspiked	21	20	> 36	0
	1 x 10^4	21	20	13.8$\pm$3.1	1283$\pm$ 56
	1 x 10^3	21	20	16.0$\pm$3.6	1302$\pm$ 49
Camembert	Unspiked	0	0	9.2$\pm$0.8 #	672$\pm$ 24
	Unspiked	21	20	> 36	0
	1 x 10^4	21	20	13.9$\pm$0.9	690$\pm$ 45
	1 x 10^3	21	20	17.2$\pm$1.6	712$\pm$ 84
Italian cheese spread	Unspiked	0	0	22.2$\pm$0.2 #	244$\pm$ 19
	Unspiked	21	20	> 36	0
	1 x 10^4	21	20	18.5$\pm$1.9	1212$\pm$124
	1 x 10^3	21	20	21.2$\pm$1.3	1158$\pm$127

(N=6)

Key

= One artefactual curve produced.

Table 5.3 Impedimetric responses of the microflora in a range of cheese suspensions (unspiked and spiked with _L. monocytogenes_) in unsupplemented GNB and GNB supplemented with 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam.

5.2.4 Development of a more sensitive impedimetric assay for L. monocytogenes

A series of experiments was next undertaken to determine whether the sensitivity of the impedimetric assay could be improved to enable zero tolerance assurance for L. monocytogenes. Various enrichment regimens were evaluated (see 2.8).

Two 50 cm^3 suspensions of 5 % and two 50 cm^3 suspensions of 10 % (w/v) cheddar cheese in GNB were prepared and stomached for 1.0 minute prior to aseptic supplementation with the selective agents, 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam. At zero time, one suspension of each concentration of cheese was spiked with a 10^{-7} dilution of a 24 h GNB culture of L. monocytogenes NCTC 11994 (which was enumerated by routine methods, see 2.4). The four enrichment vessels were then incubated at 30 °C. At 0, 2.5, 5 and 24 h, 1.0 cm^3 aliquots were removed from each enrichment vessel and, using routine conditions (see 2.6 & 2.6.1), their impedimetric responses were monitored for 36 h. The results are tabulated in Table 5.4.

Controls (data not shown) indicated that the cheese was not naturally contaminated with Listeria and unsupplemented GNB allowed the natural microflora of the cheese to produce detection curves. However, as detection curves were also produced by the natural microflora in doubly supplemented GNB, the assay was insufficiently selective to detect L. monocytogenes in either

Concentration of cheese suspension	TVC of added L. monocytogenes (CFU g^{-1} cheese)	Enrichment time (h)	Impedance DT (h) $\pm$ SD	Impedance magnitude (BUIC) $\pm$ SD
10%(w/v)	Unspiked	0	5.1$\pm$0.1	843$\pm$ 2
		2.5	2.1$\pm$0.8	794$\pm$ 14
		5.0	3.8$\pm$0.1	749$\pm$ 81
		24	8.2$\pm$0.2	566$\pm$ 25
10%(w/v)	3.6$\pm$0.2	0	4.9$\pm$0.1	968$\pm$147
		2.5	3.0$\pm$0.1	794$\pm$ 14
		5.0	2.8$\pm$0.3	756$\pm$ 15
		24	9.3$\pm$0.7	454$\pm$112
5 % (w/v)	Unspiked	0	7.8$\pm$0.2	582$\pm$ 10
		2.5	6.4$\pm$0.3	622$\pm$ 30
		5.0	6.4$\pm$0.1	622$\pm$ 79
		24	9.4$\pm$0.1	610$\pm$ 50
5 % (w/v)	3.6$\pm$0.2	0	7.5$\pm$0.2	911$\pm$330
		2.5	4.1$\pm$0.1	627$\pm$ 9
		5.0	4.0$\pm$0.6	526$\pm$ 16
		24	*	

(N=2)

Key

* = Two artefactual curves produced.

Table 5.4 Impedimetric responses of the microflora in cheese suspensions (unspiked and spiked with L. monocytogenes) in GNB supplemented with 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam after various lengths of enrichment in the same doubly supplemented medium.

5 % or 10 % (w/v) cheese suspension. This indicates that the increased concentration of cheese matrix (0.1 g cheese cm^{-3} in the 10 % (w/v) suspension, compared with 0.01 g cheese cm^{-3} in the previous direct test [Table 5.3]) and / or the increased concentration of its microflora even after 24 h selective enrichment significantly influenced the impedimetric assay.

It was therefore concluded that the impedimetric

assay needed to be modified in order to detect levels of
L. monocytogenes contamination as low as 1 organism
per 25 g. This might be achieved by diluting the
suspension from the enrichment vessels prior to
inoculation into the Bactometer wells or alternatively by
increasing the concentration of selective agents in the
enrichment medium and /or the impedimetric medium. The
former possibility was investigated in the next
experiment.

Two 50 cm^3 suspensions of 5 % (w/v) cheddar cheese in
GNB were prepared and stomached for 1.0 minute prior to
aseptic supplementation with the selective agents,
21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3}
moxalactam. At zero time, one suspension was spiked with a
10^{-7} dilution of a 24 h GNB culture of L. monocytogenes
NCTC 11994 (which was enumerated by routine methods, see
2.4). The two enrichment vessels were then incubated at
30 °C. At timed intervals, 0.1, 0.3, 0.5 and 1.0 cm^3
aliquots were removed from each enrichment vessel and
diluted to 2.3 cm^3 with freshly prepared doubly
supplemented GNB. The impedimetric responses of these
suspensions were then monitored for 36 h using routine
conditions (see 2.6 & 2.6.1). The results are tabulated in
Table 5.5.

Controls (data not shown) indicated that the cheese
was not naturally contaminated with Listeria and
unsupplemented GNB allowed the natural microflora of
cheese to produce detection curves. L. monocytogenes was
detected in the 0.1 cm^3 aliquot of spiked cheese

suspension after 24 h enrichment. When the same volume was removed after shorter periods of enrichment however, no impedimetric responses were observed during the time course of the assay. Although the data are less clear cut, larger volumes of both spiked and unspiked cheese suspensions removed after 24 h enrichment generally produced detection curves and therefore did not permit the sole detection of L. monocytogenes. Only in the case of the 0.3 cm^3 aliquot was it possible to distinguish between the spiked and unspiked cheese on the basis of DT.

It was therefore concluded that the dilution of 0.1 cm^3 of a 5 % (w/v) cheese suspension into 2.2 cm^3 of

TVC of added L. monocytogenes (CFU g^{-1} cheese)	Volume transferred from enrichment vessel	Enrichment time (h)	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
Unspiked	0.1 cm^3	0	> 36	0
		2.5	> 36	0
		5.0	> 36	0
		24	> 36	0
9.6$\pm$2.5	0.1 cm^3	0	> 36 #	0
		2.5	> 36	0
		5.0	> 36	0
		24	8.7$\pm$4.5	1159$\pm$405
Unspiked	0.3 cm^3	0	> 36	0
		2.5	> 36	0
		5.0	> 36	0
		24	28.7$\pm$0.3 (2)# b,	371$\pm$ 16
			>36 (1)b	0
9.6$\pm$2.5	0.3 cm^3	0	> 36 #	0
		2.5	> 36	0
		5.0	> 36	0
		24	4.2$\pm$4.7 #	1132$\pm$119

(Table 5.5 continued overleaf)

TVC of added L. monocytogenes (CFU g^{-1} cheese)	Volume transferred from enrichment vessel	Enrichment time (h)	Impedance DT (h) ± SD	magnitude (BUIC) ± SD
Unspiked	0.6 cm^3	0	> 36	0
		2.5	> 36	0
		5.0	> 36	0
		24	2.3±0.1 (2),ᚼ	1350±114,
			> 36 (2) ᚼ	0
9.6±2.5	0.6 cm^3	0	19.8±2.1 (2),ᚼ	855±374,
			> 36 (2) ᚼ	0
		2.5	26.3 (1) ᚼ,	984,
			> 36 (3) ᚼ	0
		5.0	> 36	0
		24	10.5 (1) # ᚼ,	1253,
			> 36 (2) ᚼ	0
Unspiked	1.0 cm^3	0	> 36	0
		2.5	> 36	0
		5.0	> 36	0
		24	15.8±14.4	713±498
9.6±2.5	1.0 cm^3	0	22.1±2.7 (2) ᚼ,	1358± 14,
			> 36 (2) ᚼ	0
		2.5	26.6 (1) ᚼ,	1230
			> 36 (3) ᚼ	0
		5.0	29.9 (1) ᚼ,	271
			> 36 (3) ᚼ	0
		24	10.2±0.2 *	1301± 60

(N=4)

Key

\# = One artefactual curve produced.

* = Two artefactual curves produced.

ᚼ = Number of curves of each type in brackets

Table 5.5   Impedimetric responses of the microflora in cheese suspensions (unspiked and spiked with L. monocytogenes) in GNB supplemented with 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam after various lengths of enrichment in the doubly supplemented medium.

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fresh selective medium after 24 h enrichment provided a suitable regimen for the detection of levels of the target organism as low as 9.6 CFU g^{-1} cheese.

Further work was then undertaken to determine the effects of using a) a larger volume of enrichment medium and b) a two stage enrichment regimen.

Two 225 cm^3 suspensions of 5 % (w/v) cheddar cheese in GNB were prepared and stomached for 1.0 minute prior to aseptic supplementation with the selective agents, 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam. At zero time these suspensions were returned to pH 7.2 by the aseptic addition of sterile 0.1 M sodium hydroxide (BDH Biochemicals) and one suspension (in enrichment vessel 2) was spiked with a 10^{-7} dilution of a 24 h GNB culture of L. monocytogenes NCTC 11994 (which was enumerated using routine conditions, see 2.4). The two enrichment vessels were then incubated at 30 °C for 48 h and at intervals the pH of each was determined. After 10 h, 2 cm^3 was aseptically transferred from the unspiked and the spiked enrichment vessels to 223 cm^3 volumes of freshly prepared doubly supplemented GNB (in vessels 3 and 4 respectively). pH monitoring of these vessels was commenced at this time. After 24 h, aliquots were removed from each of the four enrichment vessels, diluted as appropriate, and made up to 2.3 cm^3 in freshly prepared doubly supplemented GNB. Using routine conditions (see 2.6 & 2.6.1), the impedimetric responses of these diluted samples were monitored for 36 h. The data obtained on the pH profile and the impedimetric responses of the

suspension in each enrichment vessel are shown in Tables
5.6 and 5.7 respectively.

Controls (data not shown) indicated that the cheese
was not naturally contaminated with Listeria and
unsupplemented GNB allowed the natural microflora of the
cheese to produce detection curves. The concentration of
L. monocytogenes added to the spiked cheese was determined
to be 4.0 ± 0.5 CFU per 10 g, which is below the level
detectable by direct selective plating methods.

Measurement of the pH of the cheese suspensions prior
to enrichment (Table 5.6) revealed that the addition of
cheese decreased the pH of GNB from 7.2 to 6.25. This
latter value is well below optimum for L. monocytogenes.
As microbial metabolism would be expected to decrease the
pH and hence the growth rate of the target species
further, the suspensions were readjusted to pH 7.20 at the
start of enrichment. During the subsequent first 24 h of
enrichment none of the suspensions fell by more than half
a pH unit, so further pH readjustment was not considered
necessary.

Enrichment vessel	TVC of added *L. monocytogenes* (CFU g^{-1} cheese)	Enrichment time (h)	pH
One stage enrichment			
		Before pH adjustment	6.25
1	Unspiked	0	7.20
		3	7.10
		6	6.90
		8	6.85
		10	6.72
		24	6.70
		48	5.09
One stage enrichment			
		Before pH adjustment	6.25
2	0.4 ± 0.05	0	7.20
		3	7.08
		6	7.00
		8	7.00
		10	6.70
		24	6.70
		48	5.00
Two stage enrichment			
		Before pH adjustment	6.25
3	Unspiked	10	7.20
		24	6.90
		48	6.74
Two stage enrichment			
		Before pH adjustment	6.25
4	0.4 ± 0.05	10	7.20
		24	6.95
		48	4.32

Table 5.6   The pH profile of cheese suspensions (unspiked and spiked with **L. monocytogenes**) during enrichment in GNB supplemented with 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam.

Enrichment vessel	Volume transferred from enrichment vessel	Concentration of proflavine hydrochloride ($mg\ dm^{-3}$)	moxalactam ($mg\ dm^{-3}$)	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
One stage enrichment					
1 (unspiked)	$1.0 \times 10^{-1}\ cm^3$	21	20	8.5 $\pm$ 0.7	1242$\pm$ 41
	$5.0 \times 10^{-2}\ cm^3$	21	20	11.6$\pm$8.2	914$\pm$470
	$1.0 \times 10^{-3}\ cm^3$	21	20	19.0$\pm$2.9	263$\pm$ 87
	$1.0 \times 10^{-5}\ cm^3$	21	20	> 36	0
One stage enrichment					
2 (spiked)	$1.0 \times 10^{-1}\ cm^3$	21	20	6.9 $\pm$ 1.5	1267$\pm$ 16
	$5.0 \times 10^{-2}\ cm^3$	21	20	7.6$\pm$1.6	1168$\pm$ 25
	$1.0 \times 10^{-3}\ cm^3$	21	20	12.6$\pm$1.3	1198$\pm$ 61
	$1.0 \times 10^{-5}\ cm^3$	21	20	17.4$\pm$0.7 #	1349$\pm$ 77
Two stage enrichment					
3 (Unspiked)	$1.0 \times 10^{-1}\ cm^3$	21	20	13.2 $\pm$ 0.4	482$\pm$140
	$5.0 \times 10^{-2}\ cm^3$	21	20	15.3$\pm$1.6	189$\pm$ 86
	$1.0 \times 10^{-3}\ cm^3$	21	20	> 36	0
	$1.0 \times 10^{-5}\ cm^3$	21	20	> 36	0
Two stage enrichment					
4 (spiked)	$1.0 \times 10^{-1}\ cm^3$	21	20	8.6 $\pm$ 0.1	1236$\pm$ 23
	$5.0 \times 10^{-2}\ cm^3$	21	20	9.5$\pm$0.1	1223$\pm$ 17
	$1.0 \times 10^{-3}\ cm^3$	21	20	13.6$\pm$0.3	739$\pm$517
	$1.0 \times 10^{-5}\ cm^3$	21	20	18.9$\pm$0.2	1295$\pm$ 28

(N=4)

Key

\# = One artefactual curve produced.

Table 5.7 Impedimetric responses of the microflora in cheese suspensions (unspiked and spiked with _L. monocytogenes_ NCTC 11994) in GNB supplemented with 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam after 24 h one stage or two stage enrichment.

A DT of under 20 h was recorded when 1.0×10^{-5} cm^3 of spiked cheese suspension was transferred to 2.3 cm^3 of impedimetric medium after 24 h single stage enrichment, whereas the same volume of an unspiked suspension remained undetected for the 36 h of impedimetric monitoring. The pH adjustment at zero time improved the growth and metabolism of <u>L. monocytogenes</u>, 1.0×10^{-5} cm^3 of spiked cheese suspension containing sufficient organisms for detection, whereas without adjustment 0.1 cm^3 was required to enable <u>L. monocytogenes</u> to be detected after 24 h enrichment (Table 5.5). The two stage enrichment regimen inhibited the growth and metabolism of the natural microflora of cheese to a greater extent than the one stage regimen, as 1.0×10^{-3} cm^3 of unspiked cheese suspension produced an impedimetric response only in the latter case.

It was therefore concluded that enrichment may be scaled up to a volume of 225 cm^3 and selection of the target species is enhanced by a two stage regimen.

Experiments were also carried out to determine whether increased concentrations of the selective agents in the impedimetric medium would improve the detection of the target species in spiked cheese samples.

A series of 9.9 cm^3 suspensions of 5 % (w/v) cheddar cheese in GNB were prepared and were stomached for 1.0 minute. One suspension remained unspiked, while the others were inoculated with various test species (0.1 cm^3 of a 10^{-3} dilution of a 24 h GNB culture in each case). Using routine conditions (see 2.6 & 2.6.1) the impedimetric responses of 1.0 cm^3 aliquots of the unspiked

cheese suspension were monitored for 36 h in the
unsupplemented GNB and GNB supplemented with
30 mg dm^{-3} proflavine hydrochloride and 30 mg dm^{-3}
moxalactam, while those of 1.0 cm^3 aliquots of the spiked
cheese suspensions were monitored for 36 h in the doubly
supplemented GNB. Concomitantly, the impedimetric
responses of the test species alone (that is in the
absence of cheese) were monitored for 36 h in
unsupplemented GNB and GNB supplemented with 30 mg dm^{-3}
proflavine hydrochloride and 30 mg dm^{-3} moxalactam. The
results are recorded in Table 5.8.

All strains of L. monocytogenes tested, except
L. monocytogenes NCTC 10357, and all ten Listeria food
isolates could tolerate the increased concentrations of
proflavine hydrochloride and moxalactam when in the
presence of cheese. Approximately one third of these
strains were also impedimetrically detected in the doubly
supplemented medium in the absence of this foodstuff.
Unfortunately, however, increased concentrations of the
toxic agents did not improve the selectivity of the assay
by preventing the detection of L. innocua and
L. welshimeri.

Test species	Presence of cheese	Concentration of proflavine hydrochloride (mg dm^{-3})	moxalactam (mg dm^{-3})	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
None	+	0	0	7.2$\pm$0.2	524$\pm$ 13
	+	30	30	> 36	0
L. monocytogenes NCTC 10357 (serotype 1a)	–	0	0	7.3$\pm$0.1	494$\pm$ 6
	–	30	30	> 36	0
	+	30	30	> 36	0
L. monocytogenes NCTC 7973 (serotype 1a)	–	0	0	8.2$\pm$0.0	685$\pm$ 11
	–	30	30	24.8 $\sharp$	100
	+	30	30	32.0 $\sharp$	471
L. monocytogenes NCTC 5348 (serotype 2)	–	0	0	10.1$\pm$0.2	562$\pm$ 11
	–	30	30	31.4$\pm$2.6	447$\pm$ 61
	+	30	30	23.2$\pm$0.1	559$\pm$ 7
L. monocytogenes NCTC 11994 (serotype 4b)	–	0	0	10.3$\pm$0.1	669$\pm$ 8
	–	30	30	25.7$\pm$1.5	507$\pm$ 78
	+	30	30	13.3$\pm$0.2	589$\pm$ 12
L. monocytogenes NCTC 10527 (serotype 4b)	–	0	0	9.9$\pm$0.7	751$\pm$ 19
	–	30	30	> 36	0
	+	30	30	29.6$\pm$3.4	663$\pm$ 19
L. innocua NCTC 11288	–	0	0	11.0 $\sharp$	165
	–	30	30	34.9 $\sharp$	230
	+	30	30	26.6$\pm$0.4	202$\pm$ 8
L. innocua NCTC 10889	–	0	0	12.1$\pm$0.2	634$\pm$ 13
	–	30	30	> 36	0
	+	30	30	34.0$\pm$0.8	602$\pm$ 10
L. welshimeri NCTC 11857	–	0	0	9.5$\pm$0.1	747$\pm$ 6
	–	30	30	30.3$\pm$2.0	461$\pm$ 118
	+	30	30	23.6$\pm$0.7	516$\pm$ 104

(Table 5.8 continued overleaf)   (N=2)

Test species	Presence of cheese	Concentration of proflavine hydrochloride (mg dm^{-3})	moxalactam (mg dm^{-3})	Impedance DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
Food	-	0	0	9.9$\pm$0.1	589$\pm$ 12
isolate	-	30	30	23.7$\pm$1.4	657$\pm$ 83
I1	+	30	30	22.3$\pm$1.2	634$\pm$ 76
Food	-	0	0	10.5$\pm$0.3	601$\pm$ 24
isolate	-	30	30	24.2$\pm$0.2	499$\pm$ 21
I2	+	30	30	19.0$\pm$1.1	547$\pm$ 65
Food	-	0	0	10.2$\pm$0.1	614$\pm$ 31
isolate	-	30	30	> 36	0
I3	+	30	30	32.6$\pm$1.4	477$\pm$ 103
Food	-	0	0	10.6$\pm$0.2	1109$\pm$ 78
isolate	-	30	30	> 36	0
I4	+	30	30	17.3$\pm$5.8	252$\pm$ 2
Food	-	0	0	9.7$\pm$0.2	1075$\pm$ 33
isolate	-	30	30	> 36	0
I5	+	30	30	19.9$\pm$0.1	197$\pm$ 50
Food	-	0	0	10.9$\pm$0.1	1202$\pm$ 28
isolate	-	30	30	> 36	0
I6	+	30	30	21.0$\pm$1.4	279$\pm$ 94
Food	-	0	0	11.2 #	1084
isolate	-	30	30	> 36	0
I7	+	30	30	17.4$\pm$0.5	1092$\pm$ 11

(Table 5.8 continued overleaf)   (N=2)

Test species	Presence of cheese	Concentration of		Impedance	
		proflavine hydrochloride ($mg\ dm^{-3}$)	moxalactam ($mg\ dm^{-3}$)	DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
Food	-	0	0	10.4 #	1164
isolate	-	30	30	> 36	0
I8	+	30	30	18.5$\pm$0.5	252$\pm$ 1
Food	-	0	0	11.1$\pm$0.1	639$\pm$ 14
isolate	-	30	30	> 36	0
I9	+	30	30	33.8$\pm$4.2	569$\pm$ 72
Food	-	0	0	8.0$\pm$0.1	577$\pm$ 23
isolate	-	30	30	> 36	0
I10	+	30	30	32.3$\pm$3.2	489$\pm$ 42

(N=2)

Key

= One artefactual curve produced

Table 5.8 Impedimetric responses of a range of test species in unsupplemented GNB and in GNB supplemented with 30 mg dm^{-3} proflavine hydrochloride and 30 mg dm^{-3} moxalactam in the absence and presence of cheese.

Further work was then undertaken to determine whether these increased concentrations of proflavine hydrochloride and moxalactam would improve the selective enrichment regimen for L. monocytogenes.

Four 225 cm^3 suspensions of 5 % (w/v) cheddar cheese in GNB were prepared and stomached for 1 minute. Two suspensions (in enrichment vessels 1 and 2) were supplemented with 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam, and two (in enrichment vessels 3 and 4) with 30 mg dm^{-3} proflavine hydrochloride and 30 mg dm^{-3} moxalactam. At zero time the suspensions were all returned to pH 7.2 by the aseptic addition of 0.1 M sodium hydroxide (BDH Biochemicals) and those in enrichment vessels 2 and 4 were spiked with a 10^{-7} dilution of a 24 h GNB culture of L. monocytogenes NCTC 11994 (which was enumerated by routine methods, see 2.4). The four enrichment vessels were incubated at 30 °C and at intervals the pH of each was determined. After 24 h incubation samples were removed from each of the vessels and diluted as appropriate in GNB. 0.1 cm^3 aliquots of these diluted suspensions were transferred to Bactometer wells containing 2.2 cm^3 GNB supplemented with either 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam or 30 mg dm^{-3} proflavine hydrochloride and 30 mg dm^{-3} moxalactam. Using routine conditions (see 2.6 & 2.6.1), the impedimetric responses of these samples were monitored for 36 h. The data obtained on the pH profile and the impedimetric responses of the suspension in each

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enrichment vessel are shown in Tables 5.9, 5.10 and 5.11.

Controls (data not shown) indicated that the cheese
was not naturally contaminated with <u>Listeria</u> and
unsupplemented GNB allowed the natural microflora of the
cheese to produce detection curves. None of the
suspensions in the four enrichment vessels fell below pH
6.5 within the 24 h incubation period (Table 5.9).
<u>L. monocytogenes</u> could not be detected in either doubly
supplemented impedimetric medium after enrichment in GNB
supplemented with 21 mg dm^{-3} proflavine hydrochloride and
20 mg dm^{-3} moxalactam (Table 5.10). Significantly,
however, enrichment in GNB supplemented with 30 mg dm^{-3}
proflavine hydrochloride and 30 mg dm^{-3} moxalactam enabled
the detection of all except the lowest dilution of
<u>L. monocytogenes</u> tested in both types of doubly
supplemented impedimetric medium (Table 5.11).

| Enrichment vessel | TVC of added _L. monocytogenes_ ($CFU\ g^{-1}$ cheese) | Concentration of selective agents in enrichment medium | | Enrichment time (h) | pH |
		proflavine hydrochloride ($mg\ dm^{-3}$)	moxalactam ($mg\ dm^{-3}$)		
1	Unspiked	21	20	Before pH adjustment	6.21
		21	20	0	7.18
		21	20	24	6.50
2	1.2 ± 0.04	21	20	Before pH adjustment	6.20
		21	20	0	7.19
		21	20	24	6.64
3	Unspiked	30	30	Before pH adjustment	6.20
		30	30	0	7.20
		30	30	24	6.67
4	1.2 ± 0.04	30	30	Before pH adjustment	6.21
		30	30	0	7.19
		30	30	24	6.65

Table 5.9 The pH profile of cheese suspensions (unspiked and spiked with _L. monocytogenes_) during enrichment in GNB supplemented with either 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam or 30 mg dm^{-3} proflavine hydrochloride and 30 mg dm^{-3} moxalactam.

| Enrichment vessel | Volume transferred from enrichment vessel | Concentration of selective agents in impedimetric medium | | DT (h) $\pm$ SD | Impedance magnitude (BUIC) $\pm$ SD |
		proflavine hydrochloride (mg dm^{-3})	moxalactam (mg dm^{-3})		
1 (unspiked)	1.0×10^{-1} cm^3	21	20	6.4$\pm$0.0	1352$\pm$ 9
	1.0×10^{-2} cm^3	21	20	12.3 #	933
	1.0×10^{-3} cm^3	21	20	13.3 #	174
	1.0×10^{-5} cm^3	21	20	15.7$\pm$0.1	733$\pm$ 10
1 (unspiked)	1.0×10^{-1} cm^3	30	30	7.1$\pm$0.2	412$\pm$124
	1.0×10^{-2} cm^3	30	30	11.7 #	218
	1.0×10^{-3} cm^3	30	30	15.2 #	352
	1.0×10^{-5} cm^3	30	30	19.2$\pm$0.7	434$\pm$ 65
2 (spiked)	1.0×10^{-1} cm^3	21	20	2.8$\pm$0.2	1249$\pm$ 11
	1.0×10^{-2} cm^3	21	20	5.3$\pm$0.1	1254$\pm$ 41
	1.0×10^{-3} cm^3	21	20	8.6$\pm$0.3	1299$\pm$ 37
	1.0×10^{-5} cm^3	21	20	10.0$\pm$0.2	1344$\pm$ 16
2 (spiked)	1.0×10^{-1} cm^3	30	30	4.3$\pm$1.3	1048$\pm$133
	1.0×10^{-2} cm^3	30	30	7.5$\pm$1.7	1227$\pm$ 10
	1.0×10^{-3} cm^3	30	30	9.9 #	1094
	1.0×10^{-5} cm^3	30	30	12.0$\pm$0.1	1271$\pm$ 34

(N=2)

Key

= One artefactual curve produced

Table 5.10 Impedimetric responses of the microflora in cheese suspensions (unspiked and spiked with *L. monocytogenes*) in GNB supplemented with either 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam or 30 mg dm^{-3} proflavine hydrochloride and 30 mg dm^{-3} moxalactam after enrichment in 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam supplemented GNB.

Enrichment vessel	Volume transferred from enrichment vessel	Concentration of selective agents in impedimetric medium		Impedance	
		proflavine hydrochloride ($mg\ dm^{-3}$)	moxalactam ($mg\ dm^{-3}$)	DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
3 (unspiked)	$1.0 \times 10^{-1}\ cm^3$	21	20	15.4$\pm$0.0	386$\pm$ 63
	$1.0 \times 10^{-2}\ cm^3$	21	20	> 36	0
	$1.0 \times 10^{-3}\ cm^3$	21	20	> 36 #	0
	$1.0 \times 10^{-5}\ cm^3$	21	20	> 36	0
3 (unspiked)	$1.0 \times 10^{-1}\ cm^3$	30	30	15.9 #	567
	$1.0 \times 10^{-2}\ cm^3$	30	30	> 36	0
	$1.0 \times 10^{-3}\ cm^3$	30	30	> 36	0
	$1.0 \times 10^{-5}\ cm^3$	30	30	> 36	0
4 (spiked)	$1.0 \times 10^{-1}\ cm^3$	21	20	8.0$\pm$0.0	1252$\pm$ 36
	$1.0 \times 10^{-2}\ cm^3$	21	20	11.1$\pm$0.1	1197$\pm$ 2
	$1.0 \times 10^{-3}\ cm^3$	21	20	14.9$\pm$0.7	289$\pm$ 37
	$1.0 \times 10^{-5}\ cm^3$	21	20	17.3$\pm$0.3	424$\pm$ 85
4 (spiked)	$1.0 \times 10^{-1}\ cm^3$	30	30	8.9$\pm$0.1	989$\pm$ 35
	$1.0 \times 10^{-2}\ cm^3$	30	30	13.4$\pm$0.3	1091$\pm$ 6
	$1.0 \times 10^{-3}\ cm^3$	30	30	16.2$\pm$0.1	1218$\pm$ 42
	$1.0 \times 10^{-5}\ cm^3$	30	30	23.1 #	1142

Key   (N=2)

\# = One artefactual curve produced

Table 5.11 Impedimetric responses of the microflora in cheese suspensions (unspiked and spiked with L. monocytogenes) in GNB supplemented with either 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam or 30 mg dm^{-3} proflavine hydrochloride and 30 mg dm^{-3} moxalactam after enrichment in 30 mg dm^{-3} proflavine hydrochloride and 30 mg dm^{-3} moxalactam supplemented GNB.

A series of experiments was then undertaken to
determine the effects of these increased concentrations of
proflavine hydrochloride and moxalactam during enrichment
on the detection of a variety of strains of
L. monocytogenes in a range of different cheeses. One
example is recorded below.

Two 500 cm^3 suspensions of 5 % (w/v) stilton cheese
in GNB were prepared and stomached for 1 minute prior to
aseptic supplementation with 30 mg dm^{-3} proflavine
hydrochloride and 30 mg dm^{-3} moxalactam. At zero time both
suspensions were returned to pH 7.2 by the aseptic
addition of 0.1 M sodium hydroxide (BDH Biochemicals) and
one suspension (in enrichment vessel 2) was spiked with a
10^{-7} dilution of a 24 h GNB culture of L. monocytogenes
NCTC 7973 (which was enumerated by routine methods, see
2.4). After 24 h incubation at 30 °C samples were removed
from each vessel and diluted as appropriate in GNB.
0.1 cm^3 aliquots of these diluted suspensions were
transferred to Bactometer wells containing 2.2 cm^3 GNB
supplemented with either 21 mg dm^{-3} proflavine
hydrochloride and 20 mg dm^{-3} moxalactam or 30 mg dm^{-3}
proflavine hydrochloride and 30 mg dm^{-3} moxalactam. Using
routine conditions (see 2.6 & 2.6.1), the impedimetric
responses of these samples were monitored for 36 h. The
results are recorded in Table 5.12.

Controls (data not shown) indicated that the cheese
was not naturally contaminated with Listeria and
unsupplemented GNB allowed the natural microflora of the
cheese to produce detection curves. The concentration of

L. monocytogenes added to the spiked cheese was determined to be 10.0 ± 1.0 CFU per 25 g. Enrichment in GNB supplemented with 30 mg dm^{-3} proflavine hydrochloride and 30 mg dm^{-3} moxalactam enabled the detection of both dilutions of _L. monocytogenes_ tested in impedimetric medium supplemented with either the higher or the lower concentrations of selective agents, whereas the unspiked stilton sample remained undetected on all occasions (Table 5.12). All detection curves produced were of good quality.

Other strains of _L. monocytogenes_ consistently detected by the above protocol included _L. monocytogenes_ NCTC 11994, _L. monocytogenes_ NCTC 5348 and _Listeria_ food isolates I1 and I3 (see 2.1) in cheddar cheese and _L. monocytogenes_ NCTC 11994 in stilton cheese (data not shown).

It can therefore be concluded that the use of 30 mg dm^{-3} proflavine hydrochloride and 30 mg dm^{-3} moxalactam enrichment is effective for detecting very low levels of _L. monocytogenes_ (down to 10 CFU per 25 g) in cheese suspensions.

Enrichment vessel	Volume transferred from enrichment vessel	Concentration of selective agents in impedimetric medium		Impedance	
		proflavine hydrochloride ($mg\ dm^{-3}$)	moxalactam ($mg\ dm^{-3}$)	DT (h) $\pm$ SD	magnitude (BUIC) $\pm$ SD
1 (unspiked)	$1.0 \times 10^{-3}\ cm^3$	21	20	> 36	0
	$1.0 \times 10^{-5}\ cm^3$	21	20	> 36	0
1 (unspiked)	$1.0 \times 10^{-3}\ cm^3$	30	30	> 36	0
	$1.0 \times 10^{-5}\ cm^3$	30	30	> 36	0
2 (spiked)	$1.0 \times 10^{-3}\ cm^3$	21	20	12.5$\pm$0.4	1073$\pm$ 26
	$1.0 \times 10^{-5}\ cm^3$	21	20	18.6$\pm$1.4	1065$\pm$123
2 (spiked)	$1.0 \times 10^{-3}\ cm^3$	30	30	17.8$\pm$1.1 #	1056$\pm$349
	$1.0 \times 10^{-5}\ cm^3$	30	30	28.2$\pm$2.5	806$\pm$126

(N=4)

Key

\# = One artefactual curve produced.

Table 5.12 Impedimetric responses of the microflora in stilton cheese suspensions (unspiked and spiked with L. monocytogenes NCTC 7973) in GNB supplemented with either 21 mg dm^{-3} proflavine hydrochloride and 20 mg dm^{-3} moxalactam or 30 mg dm^{-3} proflavine hydrochloride and 30 mg dm^{-3} moxalactam after enrichment in GNB supplemented with 30 mg dm^{-3} proflavine hydrochloride and 30 mg dm^{-3} moxalactam.

5.3 Discussion

The constituents of foodstuffs (that is, their chemical components and naturally occurring microflora) have been found to have a significant effect on the impedimetric responses of _L. monocytogenes_. For example, milk and camembert cheese improve curve quality, whereas thick yogurts and Wensleydale cheese have detrimental effects (I. Hancock, unpublished work). As the presence of milk nearly doubles the impedance magnitude, possibly due to its calcium ion content, the incorporation of sterile milk into GNB might be used to enhance the quality of electrical curves. As poor quality electrical curves were produced in the presence of some food products, greater dilution of these foods could result in improvement of curve morphology. Dilution may have other benefits, such as reducing the influence of the foodstuff on the pH of the system and decreasing the concentration of preservatives and other anti _Listeria_ agents. Dilution would also decrease the buffering capacity and hence may result in shorter DT's (Whitley D., personal communication, 1989). However, because of the diverse effects elicited by different foodstuffs, the experiments described in this work were restricted largely to cheddar cheese suspensions.

In an impedimetric assay the normal flora of foods can influence the growth characteristics and metabolic products of the target species by reducing available nutrients, changing the pH of the medium and producing

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bacteriocins. For example the acid produced by lactic acid
bacteria, which gives them a selective advantage in
fermented dairy products (Stanier et al., 1986a; Carminati
et al., 1988), will hinder the rate of growth of
L. monocytogenes and therefore its impedimetric detection.
All lactic starter cultures examined have been found to
inhibit but not inactivate L. monocytogenes (Carminati et
al., 1988; Harris et al., 1989). Furthermore,
Pediococcus acidilactii, a lactic acid organism used as
starter culture in the production of fermented meat
products, has been shown to produce bacteriocins
inhibitory to L. monocytogenes (Hoover et al., 1989;
Farber, 1989b). Enterococcus spp., commonly used as
probiotic feed organisms for farm animals, have also been
found to produce inhibitory bacteriocins (McKay, 1990;
Arihara et al., 1991). Farrag and Marth (1989) reported
that the growth of L. monocytogenes was impeded by
psychrotrophic Ps. fluorescens, possibly due to endotoxin
release by the latter species (Walterspiel & Ronnlund,
1987). Although in this work the electrical responses of
L. monocytogenes in GNB were not found to be affected by
the addition of a range of fungal test species (see 4.4.6)
or in stilton cheese naturally contaminated with
Penicillium spp. (see Table 5.3 & 5.12), hexanoic and
octanoic acids produced by fungi have been reported to be
detrimental to the growth of L. monocytogenes (Kinderlerer
& Lund, 1992).

The physiological condition of the target organism is
a significant factor to be considered in developing a

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detection regimen. For example, the presence of a
L. monocytogenes specific phage would be expected to
affect the impedimetric responses of the host species.
Cells can also be influenced by chemical additives, such
as preservatives, and by physical food processing
procedures. Many forms of injury can be induced by these
physical and chemical means. Sublethal damage occurs
mainly to the cell membranes, impaired integrity of the
cell resulting in osmotic instability and leakage of
essential cytoplasmic components. Additionally,
cytoplasmic membrane damage can directly inhibit membrane
associated enzymes, including those necessary for cell
wall synthesis (Gilbert, 1984; Ahamad & Marth, 1990).
Damage to DNA can also occur resulting in loss of
information essential for biosynthetic and metabolic
activities (Mossel & van Netten, 1984; Ahamad & Marth,
1990).

Microbial recovery from injury is influenced by the
type of organism, the severity of the injury, the growth
conditions prior to stress, the isolation procedures and
the composition of the recovery medium (Gilbert, 1984).
Generally, injured cells are able to survive and undergo
repair if they are under no additionally imposed stress
during the initial isolation procedures. Sublethally
injured organisms may be unable to proliferate or survive
on selective media where unstressed cells would not be
affected due to their osmotic stability (Gilbert, 1984).
Severely injured cells may therefore require a period of
non selective enrichment (Brackett & Beuchat, 1989). This

could be achieved in the impedimetric assay developed for
L. monocytogenes by simply delaying the supplementation of
GNB for a suitable time period after homogenisation of the
food product under test. Additionally, detoxifying
constituents, such as charcoal, starch and sodium
bicarbonate (Mossel & van Netten, 1984), or catalase and
pyruvate (Mossel, 1986), could be incorporated into a
recovery medium.

From the investigation into the effects of
temperature shock (see 5.2.2) it was determined that
stressed cells of L. monocytogenes could be satisfactorily
detected in GNB supplemented with proflavine hydrochloride
and moxalactam and therefore a resuscitation period in
medium without these selective agents was not necessary
under the experimental conditions used. However, the use
of a two stage regimen, viz non selective enrichment
followed by selective enrichment, may need consideration
where L. monocytogenes has been subjected to a high level
of processing. DePasquale et al. (1985) reported that
bacterial injury affected impedimetric responses. This
resulted in longer DT's, and therefore led to inaccurate
enumeration of organisms when using calibration curves
produced for unstressed cells. These observations were
confirmed in the present work where heat stress delayed
the DT of L. monocytogenes (Table 5.2). The delay is
probably due to the combined effects of a reduction in
viable numbers after heat treatment and injured cells
requiring a recovery period before normal metabolism is
resumed. However, as similar delays in DT were observed in

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both supplemented and unsupplemented medium, there was no evidence of any additive or synergistic interaction between heat treatment and the selective agents proflavine hydrochloride and moxalactam.

The method used to analyse a foodstuff for L. monocytogenes depends upon the expected level of contamination. This may vary from about 10^3 CFU g^{-1} in some fresh foods to below 10^{-2} CFU g^{-1} in processed products (Mossel, 1989). The dissociation of a relatively low number of microorganisms is the first stage of any detection regimen for a foodborne pathogen. The standard procedure for solid samples is the preparation of a 10 %(w/v) suspension, followed by either blending at low speed (8,000 revolutions per minute) for 2 minutes or alternatively stomaching for different time intervals (Dickson, 1990b). No difference was found in the recovery of L. monocytogenes from beef tissues homogenised by either procedure, though blending was found to be more efficient for recovering L. monocytogenes from cheese substrates (Yousef et al., 1988; Dickson, 1990b). Differences in their physical nature and distribution of microorganisms within the foodstuffs were thought to account for these observations (Dickson, 1990b). As blending can produce aerosols, the enclosed stomacher is preferred by many groups working with pathogenic L. monocytogenes (van Spreekens & Stekelenberg, 1986; Buchanan et al., 1987; Benkeroum & Sandine, 1988; Yousef et al., 1988). In this work homogenisation in the stomacher was found to be the most efficient method of

dissociating the normal flora of cheese (see 5.2.1).
Dickson (1990b) found that increasing the stomaching time
from 0.5 to 5 minutes had no affect on the recovery of
L. monocytogenes. This notion was confirmed in the current
work, although the maximum effective time was found to be
1.0 minute (Table 5.1). Research has indicated, however,
that the recovery of the target organism may be affected
by the type of diluent used during homogenisation (Yousef
et al., 1988; Dickson, 1990b) and its temperature (Yousef
et al., 1988).

In this work the same DT was noted for varying
numbers of CFU after homogenisation in either the
stomacher or the pestle and mortar (Table 5.1). This
phenomenon may be due to equal numbers of organisms (a
constant DT) but differing extents of microbial clumping
(varying numbers of cells within a CFU) being present
after dissociation. Such clumping is a common problem
associated with the plate count method of enumeration
(Firstenberg-Eden et al., 1984).

The homogenisation procedure used also affected the
morphology of impedance curves produced, blended samples
giving curves of a consistently higher magnitude than
stomached samples or those treated in a pestle and mortar
(Table 5.1). Other workers have similarly reported that
the method of sample preparation can effect the quality of
impedance curves (Firstenberg-Eden, 1983; Arnott et al.,
1988). In this present case the difference in magnitudes
could be due to an increase after blending in the
concentration of substrates in the cheese suspension

capable of yielding ionic products. The heat generated by
this harsh procedure may have been responsible for
modifying the foodstuff itself and / or the diluent.
Alternatively, metabolites may have been released into the
medium as a result of the disruption of microbial cell
walls. The reduction in cell viability observed after
blending gives support to the latter hypothesis.

The direct impedimetric assay developed in chapter 4
was found to be capable of consistently detecting
1×10^3 CFU of L. monocytogenes g^{-1} cheese (Table 5.3),
the lower limit of detection being $1 \times 10^2 \ g^{-1}$ cheese. The
concentration of target organism per gram of foodstuff
required for this procedure is therefore similar to that
needed in traditional selective plate assays. Although the
direct impedimetric assay would permit enumeration using
predetermined calibration curves of TVC verses DT for
individual foodstuffs, the sensitivity is insufficient to
allow adherence to zero tolerance level specifications.
As scaling up is precluded because of the limits imposed
by the 2.3 cm^3 volume of the Bactometer wells, attempts
were made to modify the assay by the inclusion of a
enrichment stage.

The concentration of cells in the impedimetric
inoculum must not be above or below the threshold level
for the Bactometer, that is, detection must not occur
during the temperature equilibration period or after the
cut off point set for a positive response. To accomplish
this the optimum enrichment conditions and volume of
enrichment suspension subsequently added as inoculum to

the impedimetric medium were carefully evaluated (see 5.2.4). Copolla and Firstenberg-Eden (1988) recommended that a 5 to 10 % (w/v) suspension of foodstuff be used as inoculum in enrichment medium. In the assay developed here it was found that a 5 % (w/v) cheese suspension was required. Because of the low pH of this inoculum, the medium had to be readjusted to pH 7.2 at the start of the enrichment period to avoid any inhibitory effects on the growth and metabolism of L. monocytogenes (see Tables 5.6 & 5.9). The concentration of selective agents was also adjusted to take account of non specific binding to the organic material present in the enrichment medium and hence reduce the protection afforded to non target species in cheese suspensions. Of the Listeria test species examined, only cheese suspensions spiked with L. monocytogenes NCTC 10357 failed to produce an impedimetric response in GNB supplemented with these higher concentrations of proflavine hydrochloride and moxalactam (Table 5.8). Even though NCTC 10357 is the type strain, it is atypical, non haemolytic and has been identified as L. innocua (NCTC Colindale, personal communication, 1990). With these refinements the target organism could be detected in high dilutions of cheese suspension after 24 h single stage enrichment (Table 5.11 & 5.12), indicating that the procedure might be further optimised by a reduction in the enrichment time.

For zero tolerance assurance of foodstuffs contaminated with mixed microflora a enrichment period of 24 to 48 h has been used prior to traditional selective

plating (Kerr & Lacey, 1991), while 7 days enrichment is recommended for recovery of stressed organisms (Lovett et al., 1987; Lovett, 1988). Although further work might be necessary to improve its sensitivity (viz from 10 CFU per 25 g to 1 CFU per 25 g), zero tolerance assurance by the modified impedimetric technique developed in this study would be likely to be more rapid, the current procedure being capable of detecting both unstressed and heat stressed L. monocytogenes within 60 h. Although this time might be decreased by reducing the enrichment stage to for example 12 h, this would require inoculation of the impedimetric medium during the evening or the night, which would be impractical as few industries work triple shifts (Sharpe, 1986). This was the main reason for the investigation of the two stage enrichment regimen being discontinued (Table 5.7).

Traditional plating techniques indicated that the cheeses used in this work were not naturally contaminated with L. monocytogenes but confirmed the presence of the target organism in all spiked suspensions. Unfortunately, a number of false positive results were obtained with this procedure. On all occasions the culprit was identified by Api Zym biochemical techniques (see 2.1) as Enterococcus spp. Vicente et al. (1990) also found that media selective for L. monocytogenes were effective at isolating Enterococcus spp. and stated this was due to the similar patterns of cephalosporin sensitivity of the two genera.

While no false positive results were produced with the modified impedimetric assay, artefactual curves were

encountered. Cossar _et al_. (1990) commented that their production is almost always due to electrode disturbances. Bacterial and food sedimentation on the electrodes in the Bactometer reaction wells could therefore be the cause of the artefactual curves observed throughout this work. This problem might be reduced or prevented by the dilution or filtration of homogenised food suspensions. Alternatively, as the effect of viscosity on the mobility of ions at concentrations existing in culture media is negligible (Owens, 1985), the use of semi solid impedimetric medium might prevent the surface fouling of electrodes by food substrates. Another possible cause of artefactual curves could be metabolically evolved gas bubbles lying on the surface of the electrodes and reducing their surface area. The prevention of this type of artefactual curve would be more difficult.

The metabolic basis for the impedimetric detection of _L. monocytogenes_ was next investigated (Chapter 6).

CHAPTER 6

DETERMINATION OF THE METABOLIC BASIS OF THE
IMPEDIMETRIC DETECTION OF TEST SPECIES

6.1 Introduction

The objective of a biochemical investigation into the conditions required for impedimetric detection is to gain an understanding of the metabolic end products responsible for the electrical responses of a target organism. Such knowledge can then be used in the development of an optimal electrical medium (Zindulis, 1984).

Cossar et al. (1990) discussed the factors which significantly influence the electrical characteristics of cell suspensions. They found the electrical state to be very complex and dependent on: the monitoring frequency, the signal amplitude, the dimensions, composition and any pretreatment of the electrodes, the suspension medium and temperature, and the viability, metabolism, concentration and geometry of the cells. As commercial instruments use electronics to establish particular parameters (Firstenberg-Eden & Eden, 1984; Cossar et al., 1990) and monitoring frequency and signal amplitude are set as constants, these factors could not be evaluated with the Bactometer used in this work. The extent of the electrical change required to produce a positive detection is also important. For example, Cady et al. (1978) reported that

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the baseline had to undergo a change of 0.8 % (see 2.6.1) for a DT to be registered. As this parameter is also constant and set in the software of the Bactometer, and, as all test species produced electrical responses of sufficient amplitude to generate DT's in unsupplemented GNB, it was not investigated further. Similarly, the influence of the dimensions, composition and pretreatment of the Bactometer electrodes could not be assessed. The temperature of the impedimetric assay was constant at 30 °C and therefore temperature variation should not have had an influence except during the initial temperature stabilisation period of the Bactometer when monitoring is not undertaken. The significant criteria that remain to be investigated are therefore the medium constituents and the cell parameters.

In the work reported in this chapter an attempt was made to identify and quantify metabolites produced by different test species in order to determine the conditions which enable impedimetric detection of L. monocytogenes. The concentrations of various organic acids and of ammonia in uninoculated GNB and GNB inoculated with three test organisms used in this work, viz L. monocytogenes, Lact. lactis and Ps. fluorescens, were determined by chromatographic and spectrophotometric techniques. The optimal cultural conditions for L. monocytogenes determined in Chapter 3 were used throughout.

6.2 Results

6.2.1 Investigation of L-lactic acid produced by test species

Using a commercially available test kit (see 2.11) the concentrations of L-lactic acid were determined in freshly prepared unsupplemented GNB, GNB supplemented with 21 mg dm^{-3} proflavine hydrochloride and cell free extracts of 24 h cultures of L. monocytogenes, Lact. lactis and Ps. fluorescens grown in unsupplemented GNB (see 2.10). The results are tabulated in Table 6.1.

The presence of proflavine hydrochloride in GNB reduced the apparent concentration of L-lactic acid compared with unsupplemented GNB (Table 6.1), the strongly coloured dye influencing the spectrophotometric assay.

Of the three test species, Lact. lactis produced the greatest amount of L-lactic acid (1.322 g dm^{-3}). L. monocytogenes also produced L-lactic acid (1.071 g dm^{-3}), while Ps. fluorescens appeared to utilise the L-lactic acid present in GNB, the concentration in this cell free extract being 0.315 g dm^{-3} lower than in the uninoculated GNB.

6.2.2 Investigation of ammonia produced by test species.

Using a commercially available test kit (see 2.12) the concentrations of ammonia were determined in freshly prepared unsupplemented GNB, GNB supplemented with 21 mg dm^{-3} proflavine hydrochloride and cell free extracts

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Sample	Concentration of L-lactic acid ($g\ dm^{-3}$)	Yield of L-lactic acid ($g\ dm^{-3}$)
L-lactic acid standard solution ($0.195\ g\ dm^{-3}$)	0.196 ± 0.002	N/A
Unsupplemented GNB	0.549 ± 0.014	N/A
GNB supplemented with 21 mg dm^{-3} proflavine hydrochloride	0.411 ± 0.013	N/A
L. monocytogenes (24 h cell free extract)	1.620 ± 0.093	1.071
Lact. lactis (24 h cell free extract)	1.871 ± 0.001	1.322
Ps. fluorescens (24 h cell free extract)	0.234 ± 0.046	-0.315

(N=2)

Table 6.1 Spectrophotometric assay of the concentration of L-lactic acid in impedimetric media and cell free extracts.

of 24 h cultures of L. monocytogenes, Lact. lactis and Ps. fluorescens grown in unsupplemented GNB (see 2.10). The results obtained by comparison with the correlation graph of absorbance versus concentration of ammonium ions (Fig. 6.1) are recorded in Table 6.2.

All test species produced ammonia in GNB. Ps. fluorescens gave the greatest yield (105 mg dm^{-3}), followed by Lact. lactis (80 mg dm^{-3}) with L. monocytogenes yielding the least (32 mg dm^{-3}).

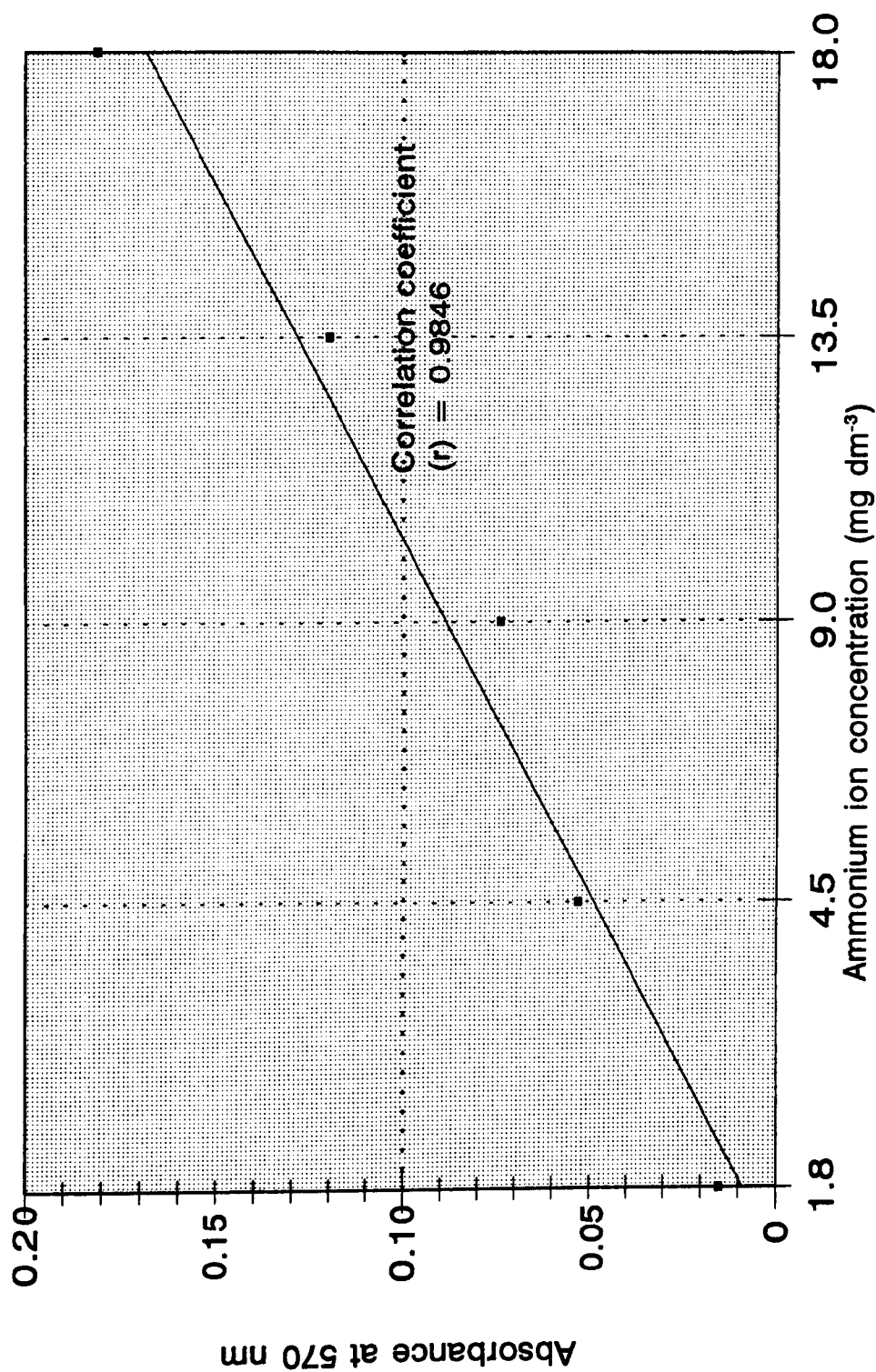The presence of proflavine hydrochloride in GNB

Fig. 6.1 Relationship between ammonium ion concentration in aqueous solution and absorbance at 570 nm. The Berthelot reaction was used to convert ammonia to chromogenic indophenol (see 2.12).

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reduced the concentration of ammonia compared with unsupplemented GNB (Table 6.2). As this toxic agent was found to affect the spectrophotometric determination of L-lactic acid and of ammonia, it was decided that this technique could not be used further in the biochemical investigation of impedimetric detection.

6.2.3 Investigation of carboxylic acids produced by test species.

Using a gas chromatograph technique (see 2.13) the concentrations of carboxylic acids were determined in freshly prepared unsupplemented GNB and cell free extracts

Sample	Concentration of ammonia ($mg\ dm^{-3}$)	Yield of ammonia ($mg\ dm^{-3}$)
Unsupplemented GNB	75 ± 5.0	N/A
GNB supplemented with 21 mg dm^{-3} proflavine hydrochloride	70 ± 2.0	N/A
L. monocytogenes (24 h cell free extract)	107 ± 17.0	32
Lact. lactis (24 h cell free extract)	155 ± 5.0	80
Ps. fluorescens (24 h cell free extract)	180 ± 1.0	105

(N=2)

Table 6.2 Spectrophotometric assay of the concentration of ammonia in impedimetric media and cell free extracts.

of 24 h cultures of L. monocytogenes, Lact. lactis and Ps. fluorescens grown in unsupplemented GNB (see 2.10). The spectra of the separated acids are shown in Fig. 6.2.

The method of Carlsson (1973) was found to be satisfactory for detecting levels of straight chain carboxylic acids of approximately 1.0 g dm^{-3} GNB or more, however the limit of detection of lactic acid was 2.25 g dm^{-3} GNB. The results obtained indicated that any acids present in the samples were at too low a concentration to be detected by the gas chromatographic technique used.

As the test protocols for L-lactic acid (see 2.11) and ammonia (see 2.12) were affected by the presence of the antimicrobial agent proflavine hydrochloride, and the chromatographic technique (see 2.13) was not sensitive enough to detect the concentration of L-lactic acid produced at 24 h, it was decided to discontinue the biochemical investigation of impedimetric detection conditions at this point. A number of factors likely to instigate the electrical responses of the test species are next discussed (see 6.3).
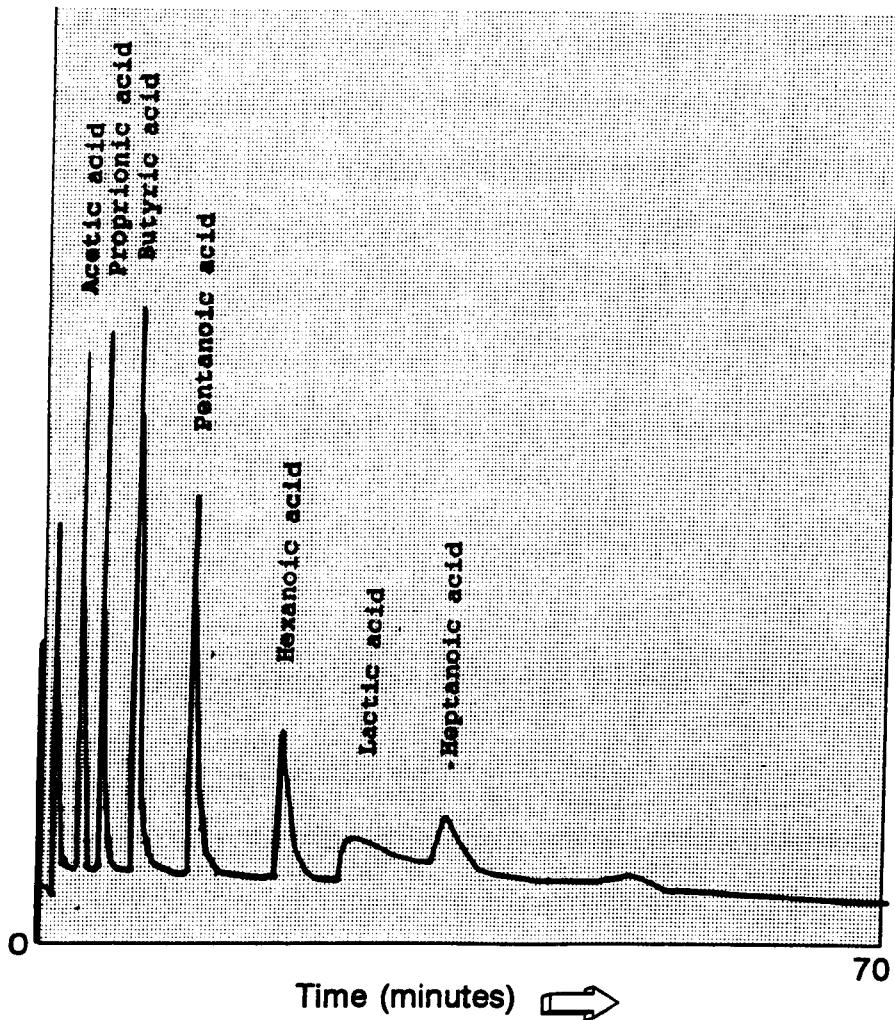
Fig. 6.2 The gas chromatographic separation of 5.2 g dm^{-3} acetic, 4.9 g dm^{-3} proprionic, 4.8 g dm^{-3} butyric, 4.7 g dm^{-3} pentanoic, 4.6 g dm^{-3} hexanoic, 4.6 g dm^{-3} heptanoic acids and 9.01 g dm^{-3} lactic acid in GNB

6.3 Discussion

As pH significantly affects conductivity (Zindulis, 1984), changes in the electrical properties of growth medium are thought to result from the accumulation of protons formed by the breakdown of constituents. Ammonium ions released on protein degradation may also be responsible for some electrical responses (Cady, 1975; Owens, 1985).

The main product of the metabolism of glucose by L. monocytogenes is L-lactic acid (Stanier et al., 1986a; Jones, 1989). Anaerobically L. monocytogenes metabolises glucose by the classic Embden-Meyerhof-Parnas (EMP) pathway producing only L-lactic acid (Benedict, 1990). Aerobically approximately 80 % of the yield of L-lactic acid is formed by the metabolism of both hexoses and pentoses (Benedict, 1990). Other products of L. monocytogenes aerobic metabolism include acetic acid and small amounts of isovaleric, isobutyric and isohydroxy acids (Pine et al., 1989). The principal metabolites expected to be produced by L. monocytogenes in GNB were therefore L-lactic acid from glucose and ammonium ions from the utilisation of proteins.

Lact. lactis is facultatively anaerobic and homofermentative, converting glucose almost exclusively to L-lactic acid via the EMP pathway (Stanier et al., 1986a). Cultures of Lact. lactis grown in glucose broth reach a final pH of approximately 4.0 to 4.5 as a result of the large quantities of acid they produce (Buchanan et al., 1974). Lact. lactis nutrition is very complex requiring

four to five B vitamins, 10 to 13 amino acids, acetate and oleate or lipoate (Buchanan et al., 1974). Under starvation conditions some pyruvate is converted to ethanol and acetate to release extra energy (Stanier et al., 1986a). The metabolites expected to be produced by Lact. lactis from GNB were therefore L-lactic acid and possibly acetic acid in the stationary phase of growth. From the catabolism of proteins ammonium ions were also expected.

Pseudomonas spp. are highly versatile nutritionally, capable of using 60 to 80 different compounds as a sole source of carbon and energy (Stanier et al., 1986b). Lactic acid and glucose can be metabolised aerobically by the majority of species. Some species produce acids from alcohols and aldose sugars especially when these substrates are provided in high concentrations. However, as Pseudomonas spp. can not grow at or below pH 6.0 (Buchanan et al., 1974), Ps. fluorescens is not likely to produce excessive amounts of acids. Certain pseudomonads accumulate ß hydroxybutyrate as an intracellular reserve material and others accumulate polysaccarides (Buchanan et al., 1974). The storing of such intracellular reserve materials would reduce the level of metabolites potentially capable of contributing to the electrical signal, however Ps. fluorescens has been reported not to produce ß hydroxybutyrate (Stanier et al., 1986b). Due to the versatile nutritional behaviour of Ps. fluorescens it is difficult to speculate about the metabolites which might generate the electrical responses of this species.

The data in Tables 6.1 & 6.2 indicate that the main metabolic products likely to instigate the electrical responses of L. monocytogenes and Lact. lactis are L-lactic acid and ammonium ions. Impedimetric detection of Ps. fluorescens, on the other hand, may depend only upon ammonium ions as the latter organism was observed to utilise L-lactic acid from GNB. This finding was not unexpected considering the organisms nutritional versatility and low acid tolerance (Buchanan et al., 1974). As the pH of GNB never exceeds the pKa of aqueous solutions of ammonia, that is 9.3 (Allison & Bird, 1964), its ammonium content will be almost entirely in the $NH4^+$ form. Such ions will therefore contribute to the electrical responses of test species.

Proflavine hydrochloride was shown to have a significant effect on the spectrophotometric determination of lactic acid and of ammonia, the presence of the coloured agent reducing the apparent concentration of these metabolites in GNB (see Tables 6.1 & 6.2). Furthermore, different extents of binding of proflavine hydrochloride to microbial receptors might result in a variable concentration of this agent in cell free extracts of cultures grown in its presence. The Berthelot reaction, utilised in the ammonia test kit, has also been reported to be affected by contact with antimicrobial agents (Kaplan, 1965; Sigma, diagnostic kit schedule). For these reasons, the use of a chromatographic procedure rather than spectrophotometry was thought to be preferable for

investigating the metabolic basis of the impedimetric detection of <u>L. monocytogenes</u>. However, problems were encountered with this technique (see 6.2.3).

Changes in pH associated with microbial growth, however, can not always be the principal cause of impedimetric responses. For example, <u>E. coli</u> produces acid metabolites in Trypticase Soy broth decreasing its pH, whereas <u>Proteus mirabilis</u> produces alkaline metabolites in Urea broth increasing pH, and yet the growth of both organisms results in a decrease of the impedimetric signal (Cady <u>et al</u>., 1978). This may at first seem surprising but pH changes could be moderated in some way by the organisms themselves. For example, they might be able to buffer changes within certain limits. It has been noticed that the cell surface charge on bacteria alters with pH, as pH increases the number of negatively charged groups also increases (Russell <u>et al</u>., 1982).

Cossar <u>et al</u>. (1990) investigated metabolically produced organic acids using chromatographic methods and ammonia using a spectrophotometric method. Unfortunately, the authors did not report the details of their findings but only commented that it was difficult to ascertain the chemical species causing the conductivity changes associated with microbial metabolism. Nonetheless, they did elucidate that in some cases an equilibrium is established where ionic species (such as organic acids) are buffered by components in the medium or organism itself. The authors also concluded that membrane bound ions affected the electrical characteristics of the

medium.

In order to quantify the biochemical conditions required for impedimetric detection it would be necessary to first determine the extent of cellular buffering and its effect on the electrical signals produced by test species. A number of components of bacterial cell walls can be identified as being capable of acting as buffers. Teichoic acids may be main source of buffer capacity in Gram positive species. Lact. lactis possesses glycerol containing teichoic acids between the peptidoglycan and the cytoplasmic membrane (Buchanan et al., 1974) and L. monocytogenes peptidoglycan contains both teichoic acids and lipoteichoic acids (Ralovich, 1984; Alvarez-Dominguez et al., 1993). The Gram negative outer membrane being lipophilic in nature would have little buffering capacity, however the cytoplasmic membranes of both Gram positive and Gram negative species contain proteinaceous constituents which are capable of buffering protons. As the cytoplasmic membrane is the main permeability barrier of a microbial cell, components retained inside this membrane are not likely to be responsible for contributing to the impedance change.

Although chromatographic techniques are available to quantify the constituents of microbial cell walls and cytoplasmic membranes capable of causing buffering (Drucker, 1981), they were not investigated in this work. It can be argued that such constituents would not influence electrical signals if they were trapped within the matrix of the peptidoglycan or on the inside of the

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cytoplasmic membrane with no contact with the impedimetric medium. The true extent of cellular buffering capacity under the experimental conditions used in this work therefore not could not be assessed as the proportion of these "trapped" constituents is unknown.
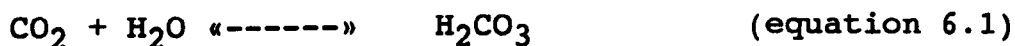
Impedance, which is sensitive to both changes in bulk conductance and in capacitance at the electrodes (see 1.5.1), was used in this work to monitor the electrical responses of test species. Using non commercial instrumentation, Cady et al. (1978) determined the relative contributions of conductance and capacitance by measuring the impedance at different frequencies (see equations 1.2 & 1.3). These authors found capacitance was the main component altered by microbial growth although measurable conductance changes also occurred. As the Bactometer gives only arbitrary values of conductance, capacitance and impedance, the three signals could not be compared directly in this work.

Microbial cell wall components could affect both conductance and capacitance. Overall conductance would be influenced by cellular buffering capacity and the cell wall and membrane bound ions themselves. Cady (1975) suggested that the charged film on the electrodes, responsible for the capacitive signal, could be composed of microbial cells as only a proportion of the electrical response remained in cell free extracts. In this case the concentration of bound ions, and the shape and size of the organism would dictate the numbers of charged species in sufficiently close proximity to the electrodes to modify
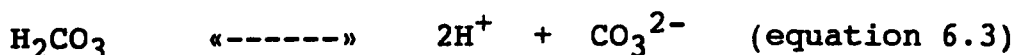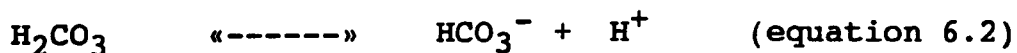
the electrical signal. Furthermore, hydrated and non hydrated ions could be excluded from the area close to the electrodes by charged microbial cells and therefore their influence on capacitance reduced.

Unfortunately quantifying the influence of microbial cell wall and cytoplasmic membrane components on the capacitive signal would not be possible. In an analogous way to their lack of functioning in cellular buffering, "trapped" components would not affect capacitance as they would not be in direct contact with the electrodes. As discussed above, the proportion of such "trapped" components is unknown and therefore the relative exposure of the electrodes to effective components could not be determined.

Other factors could also have had a significant effect on the impedimetric detection of test species. As Chapman (1980) elucidated, there are a number of possible outcomes for metabolically evolved CO_2. In culture media CO_2 mainly combines with water to form carbonic acid:

$$CO_2 + H_2O \longleftrightarrow H_2CO_3 \qquad \text{(equation 6.1)}$$

The carbonic acid is then ionised:

$$H_2CO_3 \longleftrightarrow HCO_3^- + H^+ \qquad \text{(equation 6.2)}$$
$$H_2CO_3 \longleftrightarrow 2H^+ + CO_3^{2-} \qquad \text{(equation 6.3)}$$

This acid could therefore make a contribution to the electrical responses of the test species. Carbonic acid can, in turn, combine with strong cations to form, for example, sodium carbonate. As conductivity is strongly

dependent on salt concentration (Richards et al., 1978) this might be another primary contributor to the electrical signal.

CO$_2$ can also combine directly with the NH$_2$ groups of proteins forming carbamino compounds which could decrease the buffering capacity of the system. Furthermore, CO$_2$ dissolving directly from the air into impedimetric media may cause the electrical signal to drift. Alternatively CO$_2$ can remain in culture media in simple solution, which is non ionised and therefore would not influence the electrical responses. The relative contribution to the electrical responses of test species of each of these factors could not be determined by chromatographic or spectrophotometric means.

It is also possible that a number of different acids, which were present at too low concentrations to be detected by the chromatographic technique used in this work, may make minor contributions to the electrical signals of the test species. Examples might include the small amounts of isobutyric (at approximately 1.0 x 10^{-2} g dm^{-3}) and isovaleric acids (at approximately 0.15 g dm^{-3}) produced by L. monocytogenes from glucose under aerobic conditions (Pine et al., 1989). The chromatographic technique used (see 2.13) lacked sensitivity in comparison to the spectrophotometric assay for lactic acid (see 2.11). The latter protocol permitted the detection of a 0.195 g dm^{-3} standard solution of L-lactic acid (see Table 6.1). However, as the minimum concentration of L-lactic acid detectable by the

chromatographic technique was 2.25 g dm^{-3}, this procedure could not have been used to detect the lactic acid produced by any of the three test species after 24 h culture (see Table 6.1). The limits of detection of other acid products of metabolism were also possibly too high to be of value in this research.

Other more sensitive chromatographic techniques involving extraction and derivatisation of organic acids were evaluated for use. Detection by both flame ionisation (Paquot, 1979; Darbre, 1977) and electron capture (Alley et al., 1979; Brooks et al., 1974; Darbre, 1977; Daneshvar et al., 1989; Pine et al., 1989) was attempted. However a number of problems were encountered in this work: The electron capture technique was non quantitative, lactic acid was difficult to extract, and the chromatographic spectra of timed samples from the test species did not seem to correlate. Similarly, lactic acid could not be extracted for quantification by the flame ionisation technique (I Hancock, unpublished work).

It was concluded that the determination of the conditions required for impedimetric detection of L. monocytogenes could not be achieved in the time available in this study. This was due to the complex nature of the conditions themselves and insensitivity of the available detection techniques.

CHAPTER 7

OVERALL DISCUSSION

Two selective impedimetric regimens for the detection of
Listeria spp. have been developed in this work. Firstly a
quantitative impedimetric assay using 21 mg dm^{-3}
proflavine hydrochloride and 20 mg dm^{-3} moxalactam
supplemented GNB which is capable of detecting
L. monocytogenes at concentrations greater than
10^2 CFU g^{-1} cheese within 36 h (see Chapter 5). Secondly,
a two stage detection regimen using 30 mg dm^{-3} proflavine
hydrochloride and 30 mg dm^{-3} moxalactam supplemented GNB.
This latter procedure involves preenrichnent prior to the
impedimetric assay and permits detection of the target
organism at concentrations as low as 10 CFU per 25 g of
cheese within 60 h (see Chapter 5). Preliminary data
indicate that L-lactic acid and ammonium ions are the
metabolites likely to be responsible for the electrical
responses of _L. monocytogenes_ (see Chapter 6).

7.1 Critical Evaluation of the Technique Developed.

A number of factors capable of affecting the sensitivity
of participating microorganisms and the effectiveness of
the toxic agent(s) in the selective impedimetric assay for
L. monocytogenes will be discussed further.

7.1.1 Effect of pre-assay conditions.

Among the most important pretreatment influences are the source of the organism, the composition of its growth medium and its mode of growth, for example continuous or batch culture. Additionally the pH of the growth medium and the oxygen concentration and temperature to which the organism was exposed prior to the assay can affect the phospholipid content of its cell wall and hence sensitivity to antimicrobial agents. Water activity is also important, dried bacteria being more resistant than liquid cultures (Russell et al., 1982).

As discussed in Chapter 5, organisms from processed foodstuffs can possess injuries that render them more sensitive to hostile treatments (Gilbert, 1984; Russell, 1984). When organisms have been sublethally injured, the use of toxic agents can delay or inhibit multiplication (Mossel & van Netten, 1984); pretreatment with chemical agents, for example preservatives, can therefore influence the behaviour of an organism in a subsequent assay. Surface active agents and chelating agents can increase the permeability of the cell membrane and hence allow greater access of antimicrobial agents into the cytoplasm (Russell et al., 1982). Any treatment that induces osmotically fragile forms of bacteria reduces their resistance. Examples of such damaged cells include spheroplasts (cells that have some cell wall left), protoplasts (cells with no cell wall), and mureinoplasts (Gram negative cells that have lost their outer

lipoprotein and lipopolysaccaride layers) (Russell et al., 1982).

As with all microbiological quality assessment techniques, it is clear that appropriate sampling handling techniques must be applied prior to the assay if the results are to have any validity.

7.1.2 Effect of assay conditions.

During the course of the assay itself a number of factors can influence the interaction between microorganism and toxic agent. For example, the environmental variables of temperature, pH, gaseous atmosphere and concentration of sodium chloride have been demonstrated to have an interdependent effect on the antimicrobial activity of sodium nitrate against L. monocytogenes (Buchanan et al., 1989). Changing these parameters alters the rate of access of the toxic agent to its site of action by permeation, adsorption or diffusion (Kostenbauder, 1983). Whilst Kostenbauder also commented that the temperature dependency of antimicrobial agents is very complex, Russell et al. (1982) observed that generally activity increases with increasing temperature. For example, moxalactam is more inhibitory to L. monocytogenes at 36 °C than at 30 °C (Curtis et al., 1989b).

In this work temperature, pH and concentration of sodium chloride were optimised for the most active metabolism and therefore most rapid detection of the target species and the concentration of toxic agents were adjusted to create a suitable selective spectrum. However,

antimicrobial agents which are more effective when ionised, such as the acridines, are susceptible to small pH changes and their activity is enhanced as the pH increases (Russell et al., 1982; Kostenbauder, 1983). During enrichment of food samples it was noticed that the pH decreased with time (see Tables 5.6 & 5.9). This would have the effect of decreasing both the toxicity of the selective agent, proflavine hydrochloride, and the rate of metabolism of the target species, L. monocytogenes. In order to counteract these effects the pH of stomached cheese suspensions had to be returned to pH 7.2 prior to inoculation with L. monocytogenes (see 2.8).

Adak et al. (1987b) found the carbon source affected susceptibility of microorganisms to toxic agents. Substrate limitation could have a detrimental effect on the growth of a target organism in a poor nutritional environment or where there are high numbers of competitive microflora. To avoid this problem, GNB, which itself is rich in nutrients, was further supplemented with glucose for the impedimetric assay developed in this work. It was shown that the target organism was capable of reaching its threshold level for detection in a variety of mixed cultures in this medium (Chapter 4 & 5).

As discussed in Chapter 4, the varying responses of species to individual antimicrobial agents results from inherent cytological and biochemical differences. Furthermore, as discussed in Chapter 5, the presence of a mixed flora, such as in foodstuffs, can significantly influence the antimicrobial effects of a medium as

competing microorganisms themselves may produce inhibitory substances. Access of a toxic agent to its target receptors could prove difficult if organisms were trapped within a food matrix. Solid foodstuffs are therefore routinely subjected to homogenisation prior to the selective detection of their microflora. In this work stomaching for one minute was found to be the most satisfactory method for homogenising cheese suspensions spiked with L. monocytogenes (Chapter 5).

The toxicity of a selective medium is a compromise between the inhibition of all non target species and the inhibition of the target species. If selective agents are present at too high a concentration, growth of the target species may be prevented and hence detection would not occur. Alternatively, if they are at too low a concentration, the medium would lack enough selectivity to differentiate the target organism from a mixed microflora. In this work it was found that to ensure detection of all strains of L. monocytogenes the detection of L. innocua and L. welshimeri could not be avoided (see 4.2.9). Although the latter two Listeria spp. are not recognised as human pathogens, they have no place in the food processing environment and therefore indicate process failure (Mossel, 1989).

Mossel (1986) commented that no selective medium will allow the exclusive growth of all target species for which it was designed, as in any mixed culture some non target cells will have become resistant through spontaneous mutation to the selective agent(s). Even though the false

positive detection of <u>L. innocua</u> and <u>L. welshimeri</u> could not be avoided with the detection regimen at this stage of its development, most importantly false negative results were not encountered. A number of suggestions for further work to improve the selectivity of the impedimetric medium are discussed later in this chapter (see 7.5.1).

Combinations of selective agents are often used where a spectrum of organisms is present with different resistances to single selective agents (Kostenbauder, 1983). Selective agents can react together in different ways: those acting additively are no more or less effective in combination than they are separately, while the effectiveness of individual agents is increased with synergistic combinations and reduced with antagonistic ones (Berenbaum, 1978). The two selective agents used in the impedimetric medium, proflavine hydrochloride and moxalactam, were found to act additively but minimally against the target species allowing its detection (I. Hancock, unpublished work). As the concentration of antimicrobial agents used prevented non target species from producing detection curves within the time course of the assay, whether the effect of combining the two agents was other than additive in these species could not be determined. This could form the basis for further work since a synergistic response would allow a lower concentration of one or both of the agents to be used and thus increase the speed of the assay.

Antimicrobial activity is dependent on the active agent to cell number ratio, a balance being required to

ensure saturation of the receptors in susceptible
organisms. For an adequate level of antimicrobial agent to
be maintained free in solution allowance must be made for
its depletion. If the rate of depletion of a selective
agent is known, an effective initial concentration can be
calculated (Kostenbauder, 1983). The causes of depletion
include interaction with target and non target species,
metabolism by target and non target species,
volatilisation and chemical decomposition (Kostenbauder,
1983). The latter is a possible reason for the loss of
activity observed when moxalactam was stored in solution
for a period of time (I. Hancock, unpublished work).

Partitioning between layers, for example between the
oil and water phases of a foodstuff such as cream, will
reduce contact between microorganisms in the aqueous phase
and hydrophobic antimicrobial agents (Russell et al.,
1982). For the selective toxicity of the impedimetric
medium to be maintained the selective agents have to
remain in aqueous solution in contact with both target and
non target organisms. This was the main reason for the
discontinued use of hydrophobic phenylethanol (see 4.2.5).
Both agents employed in the developed impedimetric medium,
proflavine hydrochloride and moxalactam, are hydrophilic.

Antimicrobial agents can bind non specifically with
organic matter (Russell et al., 1982; Cordier et al.,
1989). As discussed in Chapter 5, such interactions could
explain the requirement for increased concentrations of
selective agents during enrichment for L. monocytogenes
from cheese suspensions (Chapter 5). In addition, many

other substances may also interfere with the action of antimicrobial agents: Non ionic surfactants can effectively neutralise antimicrobial agents by removing them from the aqueous phase during the formation of micelles (Russell et al., 1982); certain solvents interfere with the activity of toxins (Adak et al., 1987b); cationic metal ions can increase or decrease the activity of some antimicrobial agents (Russell et al., 1982). The use of solvents except water, surfactants and metal ions was therefore avoided as much as possible in this work.

7.2 Applicability of the Developed Selective Impedimetric Assay.

The selective impedimetric assay for L. monocytogenes developed in this work has been shown to function well with mixed cultures of test species (Chapter 4) and spiked cheese suspensions (Chapter 5). Under the latter conditions the assay has been shown to be able to detect L. monocytogenes at concentrations as low as 10 CFU per 25 g of cheese and is capable of enumerating levels of L. monocytogenes detectable by direct plating via a correlation graph. However, because of their complex and variable composition, this assay may not be directly applicable to other foodstuffs. The procedure would therefore require individual evaluation for different foodstuffs and separate correlation graphs of DT and TVC would be needed for enumeration.

In addition, before commercial use, the assay would

require validating in an industrial context. Unbiased
reference (target) values would need to be obtained from
food plants which use microbiological safety assurance,
such as the Wilson Triad and HACCP (discussed in Section
1.4.1). Similarly reference values would need to be
obtained for samples that had been abused. It is important
that both sets of reference values are measured under
identical experimental conditions, otherwise conflicts of
results could arise (Mossel, 1986).

7.3 Comparison of the Selective Impedimetric Assay with Other Methods of Determining the Effectiveness of Toxic Agents.

Provided one can make the assumption that toxic agents
delay or prevent impedimetric responses by inhibiting
metabolism and growth rather than by affecting the
products of metabolism, then impedance microbiology can be
used to evaluate the suitability of such agents.

Impedance microbiology can be argued to be more
efficient than traditional microbiological methods such as
plate counts and spectrophotometric assays in determining
the effectiveness of toxic agents. The use of plate counts
necessitates the activity of the toxic agent to be
terminated at the end of a treatment period in order to
allow growth of the test organism. The separation of the
cells from the active toxic agent can be accomplished in a
number of ways. For example, filtering prior to transfer
to a solid enumeration medium. Alternatively an antidote
may be applied, but evaluation of the products of

neutralisation, which themselves must be non toxic to the target organism, can be complicated. The use of dilutions to reduce the antimicrobial agent to sub-inhibitory levels prior to enumeration has associated mathematical errors. Furthermore the diluents themselves require careful choice as some inhibit microorganisms (Russell et al., 1982). Chemical agents with intracellular receptor sites cannot be removed by these procedures, but merely further adsorption prevented. In such cases, therefore, the damaging reactions still continue (Russell et al., 1982; Gilbert, 1984).

Unless a predetermined graph of TVC versus absorbance is available, spectrophotometric assays only provide a qualitative indication of microbial sensitivity to toxic agents. Furthermore, the intrinsic problems described above in determining the TVC in the presence of a selective agent still cannot be avoided.

Impedimetric microbiology has an advantage over these traditional techniques as quantitative DT's are obtained. The DT's in the absence and presence of a particular agent are compared; any delay in the latter being attributed to the antimicrobial activity of the agent slowing microbial metabolism and hence production of detectable end products. Impedance measurements are extremely sensitive, such that within an experiment a 0.1 to 0.2 h difference in DT caused by a toxic agent is measurable and can be readily correlated to the concentration of the toxic agent. Furthermore, the changes in DT are reproducible and can be used to establish an agent's selective spectrum

against a range of test species. It can be argued therefore that the selective spectra generated by impedance technology are more quantitative and precise than data obtained via traditional microbiological methods. Furthermore this procedure avoids the problem of separating the toxic agent from the cells at the end of the treatment period. The impedimetric method is therefore ideally suited to the screening of toxic agents. The only drawback of this technology is the importance of the curve morphology and any toxic agent found to hinder electrical responses must be precluded from use. Fortunately, however, the agents used in the developed selective impedimetric assay for L. monocytogenes, proflavine hydrochloride and moxalactam, were found not to effect the electrical curves.

7.4 Comparison of the Selective Impedimetric Assay with other Rapid Methods of Detecting L. monocytogenes.

The impedimetric assay developed in this work compares favourably with other currently available rapid techniques. The preparation of samples is as simple as in traditional microbiology whereas other rapid methods, such as flow cytometry and DNA hybridisation, are more labour intensive and complex (Sharpe, 1986; Eley, 1990).

A total of 60 h (24 h enrichment followed by 36 h selective impedimetric assay) was required for the two stage detection procedure reported in this work. DNA hybridisation and the ELISA test both required a total of

51 h (48 h enrichment followed by a 3 h assay) (Dever et al., 1993; Url et al., 1993). However, as discussed later in this chapter (see 7.5.2), there are a number of ways in which the time course of the impedimetric assay might be reduced.

Several authors have compared the reliability of a number of the currently available methods for detecting L. monocytogenes. For example, Heisick et al. (1989b) observed that cultural methods detected the highest proportion of positive samples, followed by the ELISA procedure and then DNA hybridisation. However, Ralovich (1993) found the ELISA procedure to be more sensitive than cultural methods. While high levels of false negative results have been reported for all of these methods (Ralovich, 1993; Url et al., 1993), no false negatives were encountered with the impedimetric assay developed in this work.

Most rapid methods of detecting L. monocytogenes are insensitive to low numbers of cells. For example, Dever et al. (1993) found the detection limits for pure cultures to be 10^6 CFU cm^{-3} with an ELISA protocol. Similar limits of detection were observed by Ralovich (1993) using a chemiluminescent DNA probe specific for L. monocytogenes. The use of PCR has enabled lower concentrations of cells to be detected, but here the sensitivity of the assay was still limited to between 10^4 to 10^5 CFU cm^{-3} (Wernars et al., 1991; Ralovich, 1993). While immunomagnetic separation holds promise for reducing the overall assay time, this procedure can only detect a minimum of

2×10^2 cells cm^{-3} after a enrichment step. Furthermore, problems have been reported with the recognition of some serotypes of <u>L. monocytogenes</u> and high rates of non specific binding have been recorded (Skjerve <u>et al</u>., 1990; Ralovich, 1993). When used in conjunction with a enrichment step, the impedimetric assay developed in this work, however, permits the detection of concentrations as low as 10 CFU per 25 g of cheese. This detection regimen is able to detect a range of serotypes of the target organism from a variety of mixed cultures and from different types of cheese (see Chapters 4 & 5).

The initial cost of the Bactometer, at approximately £35,000, is high in comparison with the equipment required for traditional methods and some other rapid methods. However it has been reported that due to savings in materials and labour the instrument is capable of covering its own cost within three years (bioMérieux, 1991).

7.5 Suggestions for Further Work.

A number of approaches might be used in an attempt to improve the developed impedimetric regimen.

7.5.1 Strategies to improve the selectivity of the impedimetric assay

To increase the selectivity of the assay, finely divided calcium carbonate could be incorporated into GNB to neutralise the acids produced as a result of microbial metabolism. This modification, which was used by Stanier <u>et al</u>. (1986a) in a plating medium, might increase the

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buffering capacity of the medium and hence maintain the pH
longer at an optimal level for both the metabolism of the
target species and the activity of charged proflavine
hydrochloride.

As the use of antisera against <u>Neisseria gonorrhoeae</u>
was found to delay the DT of this organism by 20 h (Cady,
1975), the selectivity of the assay might be enhanced by
the addition of specific antisera against <u>L. innocua</u> and
<u>L. welshimeri</u> to preclude their detection.

Selection for <u>L. monocytogenes</u> might also be improved
by reducing the temperature of the assay to a level
selective for psychrotrophic organisms. The use of low
incubation temperatures might enable the sole detection of
haemolytic strains of <u>L. monocytogenes</u> (Junttila <u>et al.</u>,
1988), although this would clearly have the effect of
increasing the assay time and thus making the method less
useful to the food industry. Adaption of the impedimetric
method of Cady (1975) to detect charged haemoglobin,
bicarbonate and inorganic salts released from haemolysed
red blood cells, could also be used to select for virulent
strains of <u>L. monocytogenes</u>.

7.5.2 Strategies to improve the rapidity of the
impedimetric assay.

In order to improve the rapidity of the assay the
supplementation of GNB with additional growth factors for
<u>L. monocytogenes</u> could be investigated. Possible examples
include iron (Kramer & Jones, 1969; Cowart & Foster, 1985;
Shelef, 1989), glutamine (Kramer & Jones, 1969), serum and

blood (Leighton, 1985). Methods of increasing carbon dioxide concentrations in the reaction wells could be examined as this gas stimulates the growth of L. monocytogenes. Examples include the use of the oxyrase enzyme (Yu & Fung, 1992) or the addition of a layer of sterile paraffin over the impedimetric medium.

The constituents of GNB could also be adjusted to increase the concentration of the precursors of the main ionic products required for electrical detection (see Chapter 6). This might have the additional effect of improving curve morphology.

As extensive dilution of enrichment culture was required in the two stage detection regimen (Chapter 5), the dilution of the inoculum from the enrichment culture and the concentration of selective agents in the impedimetric medium could both be adjusted to set the cut off time for a positive detection at 10 h instead of 36 h. Alternatively, larger (for example 500 cm^3) impedimetric reaction wells could be manufactured and therefore increase the sensitivity of the assay without the need for enrichment.

7.5.3 Strategies to improve the recovery of the target species from foodstuffs prior to the impedimetric assay.

Work could also be undertaken to increase the recovery of L. monocytogenes from food samples using physical methods prior to the detection regimen. For example, sterile sand could be employed to aid disruption of solid food samples (Dickson, 1990b). To increase the recovery of

L. monocytogenes during stomaching a range of diluents could be tested as some have been found to be more effective than others (Yousef et al., 1988; Dickson, 1990b). Diluents supplemented with potassium hydroxide (Lovett et al., 1987) and at a temperature of 40 °C instead of 20 °C (Yousef et al., 1988) have been observed to be most useful.

Alternative recovery methods might make use of partition coefficients, as L. monocytogenes is found in the water phase rather than the lipid phase (Olsen et al., 1988). Dominguez et al. (1989) reported a method based on centrifugation to increase the recovery of L. monocytogenes from milk. Using this technique the target organism could be concentrated in cream and sediment layers. A microfilter technique could also be used to concentrate low levels of target organisms prior to an impedimetric assay (Cady, 1975).

The target organism might also be extracted and concentrated directly from food homogenates, resuscitation media or enrichment broths using an immunomagnetic separation technique developed by Skjerve et al. (1990). Providing current problems with non specific binding were overcome, this procedure could be used to retrieve L. monocytogenes when at concentrations greater than 2×10^2 cells cm^{-3} from heterogeneous mixed cultures and then the immunomagnetic beads added directly as inoculum into the Bactometer wells.

7.5.4 Strategies to develop confirmatory tests for L. monocytogenes.

Confirmatory tests for L. monocytogenes could be included in the impedimetric assay itself. For example the addition of a L. monocytogenes specific phage should delay the DT of a positive sample compared to a control well lacking a phage. This strategy was used by Pugh et al. (1988) for the detection of salmonellas.

Similarly adaptation of the Strep. pyogenes detection technique of Cady (1975) may allow the identification of pathogenic L. monocytogenes in a blood enriched impedimetric medium by lack of haemolysis in a duplicate well containing antiserum against haemolysin. Use might also be made of the possible correlation of L. monocytogenes haemolytic ability with sensitivity to nisin (Mohamed et al., 1984).

Alternatively samples could be removed from the reaction wells when the DT was reached and transferred to a traditional plating medium such as McBride Listeria agar. This would enable differentiation of L. innocua, L. welshimeri and non haemolytic L. monocytogenes from haemolytic strains of L. monocytogenes.

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