DEVELOPMENT OF MOLECULAR BIOLOGICAL METHODS FOR MONITORING THE GROWTH OF *LISTERIA MONOCYTOGENES.*

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by Michael George Milner.

March 1996.

CHAPTER 4. OPTIMISATION OF NUCLEIC ACID EXTRACTION TECHNIQUES FOR THE DETECTION OF *L. monocytogenes*.

CHAPTER 5. THE RELATIONSHIP BETWEEN RNA/DNA RATIOS AND THE GROWTH OF *Listeria monocytogenes*.

Summary.

Oligonucleotide probes and PCR primers were designed to detect *Listeria monocytogenes* 16S rRNA and rDNA. These demonstrated persistent cross reactivity to a *Listeria innocua* strain. Nucleic acid sequences from the 16S rRNA gene of a number of *Listeria* spp. showed that reliable identification of *L. monocytogenes* could not be achieved by using oligonucleotides which target the V2 region (143-220bp.) of the gene. Nucleic acid sequence analysis indicated that 16S rRNA gene sequences from clinical isolates were of increased diversity with respect to culture collection strains. Restriction endonuclease analysis of *L. monocytogenes* chromosomal DNA indicated that there were 5 copies of the 16S rRNA gene.

Fluorescent techniques were developed to quantify nucleic acids both in whole cells and nucleic acid extracts. The amount of of nucleic acids extracted by a variety of physical and enzymatic methods of cell disruption was assessed by these methods. The aim was to extract both RNA and DNA of a high structural integrity to permit both fluorescent and oligonucleotide probing assessment of RNA/DNA ratios. Whilst these physical disruption methods evaluated released a large quantity of fluorescent material from *L. monocytogenes* cells, variability was high, and simultaneous recovery of RNA and DNA was not achieved. On the contrary, some enzymatic disruption methods recovered both RNA and DNA, but in reduced amounts with respect to physical lysis methods. A lysis protocol incorporating proteinase K was used thereafter. Nucleic acid extraction from milk seeded with *L. monocytogenes* cells recovered DNA only.

Quantitative oligonucleotide probing using the rRNA directed probe MV9RP2 and rDNA probe pA permitted assessment of nucleic acid ratios, in conjunction with fluorescent methods, under various growth conditions. Fluorescently linked oligonucleotide *in-situ* hybridisation studies were successful with domain-specific probe EUB338 but not with *L. monocytogenes* specific oligonucleotide MV9RP2.

In shake flask culture of *L. monocytogenes*, nucleic acid ratios increased rapidly during exponential growth, and fell rapidly after a rapid pH mediated decline in viable counts. Maintenance at neutral pH resulted in a significant population for more than 17 days. Nucleic acid ratios increased rapidly during exponential growth, but after a stationary phase had been attained, decreased gradually. Nucleic acid ratios followed similar patterns to those reported for other organisms using different techniques.

Nucleic acid ratios were measured by fluorescent and oligonucleotide probing methods when *L. monocytogenes* was cultured at a number of growth rates in a minimal media. This showed that RNA/DNA ratios increased with respect to growth rate. However, further delineation, for example using continuous culture methods would be required to provide increased confidence in the relationship found.

Injury of *L. monocytogenes* cells by acid showed that addition of HCl to both stationary and mid-exponential cultures in a minimal medium had little effect on proliferation of the organism. Nucleic acid ratios were slightly perturbed by acid addition.

Nucleic acid ratios decreased significantly after heat injury of *L. monocytogenes*. The decrease observed was greater in cultures grown at 25°C, compared to those grown at 40°C prior to heat injury. Cultures grown at 40°C recovered significantly quicker than those grown at 25°C. Recovery was not observed in heat injured cells inoculated into a minimal medium and addition of pyruvate had no discernible effect on the rate of recovery.

ACKNOWLEDGEMENTS.

I would like to thank Prof. Jon Saunders and Dr. Alan Mc.Carthy for their encouragement and readily available advice over the past three years which has been much appreciated. At the Bedford end of things I would like to thank Steve Parry, Duncan Talbot and Mark Metcalfe for organising my visits and allowing me to appreciate that whilst the South does have warm, flat beer, there are some excellent curries in those tepid regions. Many thanks to Fiona Watson (Dept. Biochemistry) for showing me how not to destroy the spectrofluorometer and allowing me unbridled use of the thing. A big thank you to Misk (Ian Miskin) for help with DNA sequence analysis and without whom I would have destroyed the Unix terminals on the ground floor.

Now then, [this is without doubt, the hardest part of the thesis to write, and the cause of many sleepless nights] the 'witty bit'.

A big "thank-you" to everyone at DOGAM for a most enjoyable and unique environment. To mention Dept. celebrities past and present would take many pages, (more than my delicate fingers could stand) and I do not wish to cause offence by being selective. (The Mount Vernon) To those who endured the sonic burst of noise from the Tony Quail Band; (you know who you are, and you will never make it to Vaudeville) thanks for turning up/shouting/throwing things. The members of laboratoire 8.02, past and present are a very strange bunch, and long may it remain so. Where else, I wonder has there existed a glitter ball, 'what time is Love' indicator, 'disco'-effect tape recorder, free-fall radio testing, Knobby the Fridge and many strange noises/smells?! The Future of British Science. Anyway, to Trix, (Richard Hastings); Dr. Love aka. Little Fella aka. Will Hiorns; Mincer, (Derry Mercer); Coz, (Corrine Whitby); Pozzer, (Paul Loughnane); Kev Mobbs, the most tolerant cricket coach in 'da werld'; and last but by no means least Big Fella, (Mick West: life threatening violence-a speciality) have a good 'un from me, and keep the "lamp of lunacy" burning bright.

Finally, I wish to thank Helen for considerable understanding and support, particularly when it wasn't all smiles back at the shack, and tolerating many occasions of 'another late night at the office'. Many thanks to Mum and Dad for providing 5 months R&R (roof and rations) whilst battering this computer.

I hope to rid myself of a certain 'fishy' presence that had dogged my existence at Liverpool.

I shall continue to watch and wait.

ABBREVIATIONS.

min.	minutes
h.	hours
M	moles.l^{-1}
mmol.	moles.l^{-1} x 10^{-3}
(w/v.)	weight per volume
(v/v.)	volume per volume.
g	relative centrifugal force.
rpm.	revolutions per minute.
cfu.	colony forming units
Ti	irreversible melting temperature of nucleic acid duplex (Sambrook et. al., 1989)
TSYGB	tryptone soya broth supplemented with glucose and yeast extract
TSYGBP	TSYGB + 1% (v/v.) pyruvate solution.
TM	Trivett and Meyer (1971) minimal media.
TMP	TM + 1% (v/v.) pyruvate solution.
NAB	nutrient agar supplemented with defibrinated horse blood.
nt.	nucleotides.
bp.	base pairs.
kb.	kilobases

N.B. Standard chemical symbols were used throughout.

" To Mr. and Mrs. Leslie Hammond "

"...and the memory of liver sausage slurry, liquid whole egg and meat emulsion."

CHAPTER 1.

INTRODUCTION

Chapter 1. INTRODUCTION

1.1.

Listeria monocytogenes is a Gram-positive, non-spore forming, facultatively anaerobic rod. It is oxidase negative, catalase positive and produces a β-haemolysin, the activity of which may be observed on agar plates supplemented with bovine or equine blood. Growth is observed between -0.4 and 50°C (Farber and Peterkin 1991); peritrichous flagella are expressed between 20 and 25°C and confer tumbling motility. A wide variety of media sustain growth between pH 4.5 and 7.0 (Parish and Higgins, 1989). The non-fastidious nature of the pathogen is such that growth is supported in a wide variety of environments, in particular foodstuffs. Consequently, the pathogenic potential is great, and recently there has been a dramatic increase in research on this organism. Excellent reviews by Lamont *et. al.*, (1988) and Farber and Peterkin (1991) provide comprehensive accounts of the microbiology, pathogenicity, ecology and detection of this pathogen up to respective dates of publication. This thesis intends to outline the physiology, pathogenicity and ecology of *L. monocytogenes* with emphasis on relevant studies published in the last five years.

1.2. Taxonomy.

The taxonomic position of the genus *Listeria* has been examined by numerical taxonomy, of morphological, physiological and biochemical characteristics and includes studies in nucleic acid composition (Jones and Seeliger, 1987; Rocourt, 1988; Seeliger, 1984 and Collins *et. al.*, 1991). The genus *Listeria* comprising the species *L. monocytogenes, L. innocua, L. ivanovii, L. grayi, L. seeligeri, L. murrayi* and *L. welshimeri*. Of the above, only *L. monocytogenes* is a human pathogen, although both *L. ivanovii* and *L. seeligeri* have been reported to be pathogenic in sheep and horses (Farber and Peterkin, 1991). There is evidence from enzyme electrophoresis and DNA studies which indicate that *L. monocytogenes* is not a homogenous species, that may be evolving along divergent lines, concurrent with serotype, flagella proteins and 23S

rRNA sequences (Rasmussen *et. al.*, 1995). Traditionally, the taxonomic position of the genus is as a member of the family *Corynebacteriaceae* (Stuart and Pease 1972). The genus is closely related in taxonomic terms on the basis of polyphasic study to *Jonesia* and *Brochothrix*. In turn, these genera are between genera *Lactobacillus* and *Bacillus* with a more distant taxonomic relationship to *Streptococcus, Lactococcus* and *Enterococcus, Staphylococcus, Kurthia, Gemella* and *Erysipelothrix* (Farber and Peterkin, 1991). The taxonomy of the genus determined by 16S rRNA phylogeny (Collins *et. al.*, 1991) is shown in Fig. 1.2.

1.3. Pathogenicity.

L. monocytogenes expresses a number of genes associated with pathogenicity. *L. monocytogenes* adheres to host cells (often host macrophages or other phagocytes) with the aid of an adhesin protein, p60. Expression of the *p60*, *plc*, *hlyA*, *prtA* and *plc* genes are probably regulated by the *prfA* gene product (Kuhn and Goebel 1989). The bacterial cell is enveloped by host cell phagocytosis and protected by the product, a lecithinase, of the *plcA* gene (Ruhland *et. al.*, 1993). *L. monocytogenes* is released from this vesicle, the phagosome, by expression of listeriolysin O, a thiol-activated toxin from the haemolysin A gene. Subsequently, the bacterial cell divides inside the host cell, evading host-mediated detection. *L. monocytogenes* cells are forced from one host cell to another by actin filaments. Actin synthesis occurs in the host cell by a metalloprotease, encoded by the *prtA* gene (Domann *et. al.*, 1991) and is polymerised into 'rockets' by the *actA* gene product (Kocks *et. al.*, 1992). The bacterial cell is enveloped by the succeeding host cell and actin rockets removed by a phospholipase encoded by the *plc* gene (Mengaud *et. al.*, 1991; Portnoy *et. al.*, 1992).

Disease caused by *Listeria monocytogenes* occurs predominantly in young infants, geriatrics and patients with depressed T-cell mediated immunity, for example: those suffering from alcoholism, diabetes mellitus, cardiovascular and renal diseases. Listeriosis may cause bacteraemia, endocarditis and bacterial meningitis. Mortality

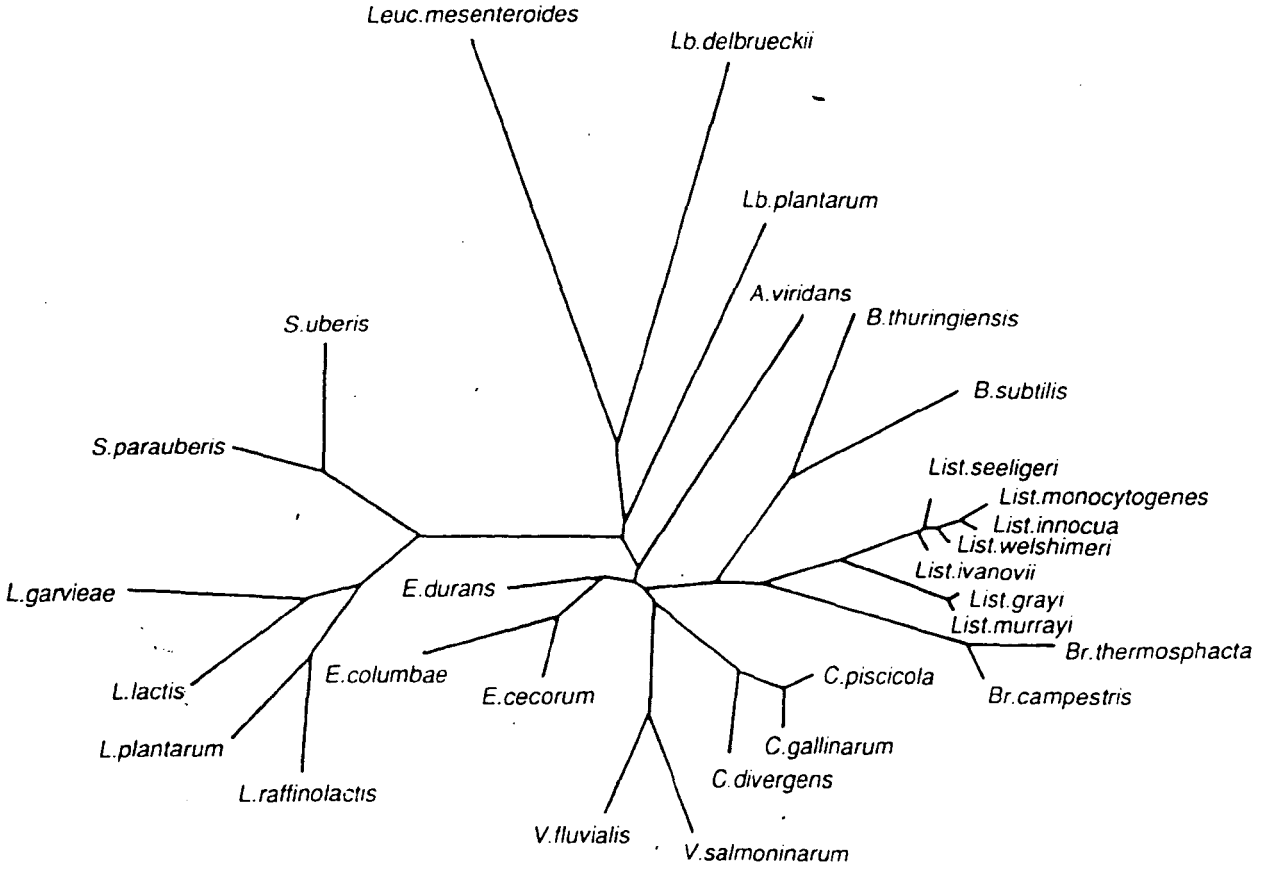


Figure 1.2. Unrooted tree or network showing the phylogenetic interrelationships of listeriae and other low G + C content Gram-positive taxa. Abbreviations: *A., Aerococcus; B., Bacillus; Br., Brochothrix; C., Carnobacterium; E., Enterococcus; L., Lactococcus; Lb., Lactobacillus; Leuc, Leuconostoc; List., Listeria; S., Streptococcus; V., Vagococcus.* From: Collins *et. al.*, (1991).

directly arising from listerial infection varies from 9% to 57% observed in patients suffering from cancer and listerial brain abscesses, respectively (Farber and Peterkin, 1991). Neonatal listeriosis usually manifests itself by premature, or low birth weight infants who become symptomatic either 1 day after birth (early onset) up to 2 weeks after birth (late onset). Early onset listeriosis is often disseminated, but concentrated in the placenta and liver, whilst late onset listeriosis predominantly causes meningitis. Mortality in both instances is predominantly due to pneumonia and respiratory failure. Carrier mothers may be asymptomatic, but carrier status does not necessarily confer foetal infection (Farber and Peterkin, 1991).

1.4. Epidemiology.

Listeriosis occurs at a rate of 2-15 cases per million head of population world-wide (Farber and Peterkin, 1991). Point of entry of the organism and other members of the genus into the food chain are traced by several techniques. The principal method of late has been by serology. There are 13 reported serotypes of *L. monocytogenes* based on O and flagella (H) antigens (Seeliger and Jones, 1986) and analysis of haemolysis by the CAMP test (Christie *et. al.*, 1944; McKellar 1994). Principally, disease is caused by serotype 4b in Europe and serotypes 1/2a and 1/2b in North America. However, this does not resolve, or indeed conclusively identify all pathogenic strains (McKellar, 1994). Consequently, alternative, or complementary typing systems have been proposed employing phage typing (McLauchlin *et. al.*, 1986; Marquet-Van der Mere and Audirier, 1995), isoenzyme patterns (Fenlon *et. al.*, 1995; Harvey and Gilmour 1994; Nørrung and Skovgaard, 1993 and Baxter *et. al.*, 1993), monocine typing (Farber and Peterkin 1991), acrylamidase (Bille *et. al.*, 1992). Nucleic acid techniques have been developed to permit *L. monocytogenes* typing. These examine chromosomal DNA polymorphism's between strains (Farber and Addison, 1994, Brosch *et. al.*, 1994 and Neiderhauser *et. al.*, 1994) ribosomal DNA (Czajka *et. al.*, 1993 and Jensen *et. al.*, 1993) or specific genes associated with *Listeria* function (Rasmussen *et. al.*, 1995). The most convenient method of determining epidemiology would probably comprise

alternative tests in conjunction with serology. Alternative typing systems described above require rigorous standardisation to ensure reliable results (Jensen *et. al.*, 1993; Neiderhauser *et. al.*, 1994). Data produced requires care in interpretation which would require personnel training to derive maximum benefit from these techniques.

1.5. 1. Growth Requirements and Physiology.

Thermostability.

L. monocytogenes has been observed to grow between -0.4 and 50°C (Juntilla *et. al.*, 1988 and Walker and Stringer, 1987). Enhanced cryotolerance shown by *L. monocytogenes* is possibly conferred by accumulation of glycine betaine, particularly where Na^+ ions are in abundant supply (Ko *et. al.*, 1994). This phenomenon proves a convenient strategy for selective enrichment of the organism.

Recently, the role of culture conditions prior to, and in repair after heat injury has been studied in detail. These showed that cultivation between 37° and 42°C for 30min to 5 h. conferred increased thermal resistance at 52-58°C (Stephens and Jones, 1993; Smith *et. al.*, 1991) with respect to lower culture temperatures. Studies have shown that saturated fatty acid content in cytoplasmic lipids decreases as growth temperature increases (Beuchat and Worthington 1976). As culture temperature increases, this reputedly reduces membrane viscosity (Smith *et. al.* 1991). Increased thermotolerance has been connected with NaCl concentration in the media used (Anderson and Jones 1991; Stephens and Jones 1993). Formulation of repair broths to maximise recovery of injured *L. monocytogenes* cells has indicated that addition of catalase, simple carbon sources, Mg^{2+}, Fe^{2+}, yeast extract, and Ca^{2+} all aid repair (Patel and Beuchat 1995; Busch and Donnelly 1992; Kihm *et. al.*, 1994, respectively). Presumably a supply of a simple carbon source and yeast extract (high in B-vitamin content) will aid recovery by reducing the maintenance energy demand of the cells. Kihm *et. al.*, (1994) proposed that heat or chemical injury was due to disruption of cell wall polymers, sufficient to release ions from the cell. However, study of cellular repair

by Bunduki *et. al.*, (1995) suggested little leakage of proteins or amino acids from *L. monocytogenes* cells after heat or chemical injury, and addition of an inhibitor of cell wall synthesis had little observed effect on cellular repair and recovery. A Fe^{2+} requirement was proposed for haemolysin, cytochromes, catalase and peroxidase (Busch and Donnelly 1992). Heat injury has been reported to have negligible effect on superoxide dismutase levels (Pedras *et. al.*, 1994) for which Mn^{2+} ions are a cofactor. No requirement for Mn^{2+} has been studied. Increased levels of oxidative phosphorylation were observed in *L. monocytogenes* cells undergoing repair and recovery (Bunduki *et. al.*, 1995). Divalent magnesium ion concentration has been reported as an important factor in maintenance of functional ribosomal units (Hapke and Noll 1976; Noll and Noll 1976). Loss of Mg^{2+}, due to heat injury, may cause dissociation of 70S ribosomal particles that, in turn are susceptible to heat degradation (Stephens and Jones 1993). It is proposed that increases in external NaCl concentration causes cellular dehydration which confers increased thermotolerance. Consequently, cellular Mg^{2+} concentration is increased relatively which results in increased bonding affinity of ribosomal particles that in turn, become more heat-resistant (Stephens and Jones 1993; Anderson *et. al.*, 1991). Increased thermostability of 30S ribosomal subunit (and subsequently 70S ribosomes) is also conferred by exposure to sub-lethal temperatures prior to heat destruction in various medium (Stephens and Jones 1993). This study raises doubts concerning the role of heat shock proteins in *L. monocytogenes*. However, research by Smith and Marmer (1995) indicates the activity of a protective protein which confers increased *L. monocytogenes* thermostability.

Mathematical models have attempted to describe effects of pH, culture temperature, NaCl concentration and heating rate on survival of *L. monocytogenes* during heating (Linton *et. al.*, 1995; Stephens *et. al.*, 1994). Models have been supported by experimental studies and show that thermotolerance is conferred at heating rates between 0.7 and $5°C.min^{-1}$. Study of D values (time at a given temperature to reduce cell numbers by 1 log cycle) and z-values (increase in

temperature required to reduce time D by a factor of 10) have been conducted on a variety of foodstuffs. Data produced and a predictive equation showed maximum values of D and z were 16.7min in ground beef and 10.4°C in fermented sausage (Miles and Mackey 1994).

pH.

Growth of *L. monocytogenes* has been observed between pH4.5 and pH7.0 (Farber and Peterkin, 1991), and growth initiated at pH4.7 in the presence of acetic acid (Young and Foegeding, 1993). Intracellular pH has been shown to be maintained significantly above that of the surrounding environment in acidic conditions (Ita and Hurkins, 1991 and Young and Foegeding, 1993). *L. monocytogenes* is capable of considerable persistence at pH3.5, albeit with reduced listeriolysin expression (Datta and Kothary, 1993), although survival time is dependent on the type of acid used (Ita and Hurkins, 1991). Growth inhibition is reputedly due to cytoplasmic accumulation of undissociated acid and consequent toxic effects. Little research has been published regarding effects of alkaline pH on *L. monocytogenes* growth and survival.

Water activity.

Water activity is a measurement of the available water in a given matrix. Minimum water activity (a_w) which permits *L. monocytogenes* growth initiation has been found to be 0.90 in a complex media at 30°C (Farber *et. al.* 1992). This value has been shown to increase as growth temperature decreases. This is lower than many other commonly occurring food pathogens, for example *Salmonella* spp., 0.95; *Campylobacter*, 0.92 and *Vibrio parahaemolyticus*, 0.94 (Lynch and Hobbie, 1988).

Osmotic stress.

Considerable resistance to sodium chloride induced osmotic stress is conferred by specific compounds. In a complex media growth initiation was observed at up to 10%(w/v.) NaCl content (McClure *et. al.*, 1989). In a defined media growth was

significantly reduced at > 0.5% (w/v.) NaCl and impaired > 3% (w/v.). Addition of betaine and carnitine to the media increased NaCl tolerance to 3%(w/v.) (Beumer *et. al.*, 1994). Uptake of L-carnitine (which is metabolically inert in *L. monocytogenes*) has been shown to be mediated by a specific ATP-dependent membrane transport system that confers osmotolerance (Verheul *et. al.*, 1995). Sodium chloride tolerance was also demonstrated to be a temperature dependant phenomenon; at lower temperatures NaCl tolerance was decreased, possibly due to increased maintenance energy demands (Beumer *et. al.*, 1994). Supplementation of minimal media with betaine and peptone conferred similar salt tolerance to that exhibited by *L. monocytogenes* in a complex medium. Peptone was shown to be pooled by the cell, presumably as a source of amino acid precursors which coincidentally contributed to osmoregulation (Amezaga *et. al.*, 1995). In addition, cellular accumulation of glycine betaine has been shown to increase the Na^+ ion tolerance of *L. monocytogenes* (Ko, *et. al.*, 1994).

Atmosphere.

Both aerobic and anaerobic conditions support growth of *L. monocytogenes*, although growth rate and yield are reduced under anaerobic conditions in complex media (Buchanan and Philips, 1990). Anaerobic growth has been observed on a reduced range of carbon sources than support aerobic growth (Pine *et. al.*, 1989). This study suggested that only hexose and pentose sugars supported growth under anaerobic conditions.

Carbon source.

Pine *et. al.*, (1989) and Premaratne *et. al.*, (1991) demonstrated that many simple, and complex carbon sources, excluding sucrose, support growth of *L. monocytogenes* under aerobic and anaerobic conditions. In the presence of amino-sugars such N-acetyl glucosamine and cell wall components, growth is enhanced relative to glucose as sole carbon source (Premaratne *et. al.*, 1991).

Nitrogen source.

An efficient di- and tri-peptide transport system has been shown to exist in *L. monocytogenes* (Verheul *et. al.*, 1995). This has a high affinity for proline-containing amino acids and effects peptide provision for growth and osmoregulation. Seven amino acids have been shown to be essential for *L. monocytogenes* growth in defined media (Premaratne *et. al.*, 1991). However, supplementation of L-histidine instead of L-glutamine significantly improve the growth rate in a defined medium (Trivett and Meyer, 1971; Jones *et. al.*, 1995).

Iron.

In common with other members of the genus, *L. monocytogenes* contains an efficient membrane-bound iron reducing enzyme, which has a broad specificity for both organic and biological sources of iron (Cowart and Foster 1985; Kemp *et. al.*, 1993). Enzyme activity is affected by external pH, temperature and presence of oxygen. Activity is apparently unaltered by the presence of Fe^{2+} in media. However, it is not clear if an iron-limited culture media was used in this study (Deneer *et. al.*, 1995).

Summary.

In view of the above findings, it is not surprising that *L. monocytogenes* will grow in a variety of complex laboratory media. These commonly include brain heart infusion broth (Ko *et. al.*, 1994 and Amezaga *et. al.*, 1995), Luria broth (Deneer *et. al.*, 1995) and tryptone soya broth (Deneer *et. al.*, 1995 and George and Lund 1992). Supplementation of tryptone soya broth with yeast extract and glucose decreases the lag phase and increases the growth rate compared to other complex medium (George and Lund, 1992). Growth in a semi-defined media containing 9 amino acids and casitone was reported to be comparable to that in a complex media (ter-Steeg and Pieterman, 1991). In defined media, a buffered minimal salts media is supplemented with the amino acids, vitamins and growth factors required for growth (Friedman and

Roessler, 1961; Welshimer, 1963; Trivett and Meyer, 1971; Ralovich *et. al.*, 1977; Siddiqui and Kahn, 1989 and Premaratne *et. al.*, 1991). The type of iron source contained in a minimal medium has been shown to be influential in supporting *L. monocytogenes* growth (Cowart and Foster, 1985). Comparative study of several defined medium reputedly suited for *L. monocytogenes* growth showed Trivett and Meyer (1971) media to be the most suitable, but none equalled growth yield and maximum specific growth rate (μmax) observed in a complex media (Jones *et. al.*, 1995). Growth of some strains of *L. monocytogenes* over successive sub-cultures in minimal medium is reported to be problematic (Premaratne *et. al.*, 1991; Jones *et. al.*, 1995).

1.5.2. Occurrence, growth and survival of *Listeria* in foodstuffs.

L. monocytogenes is found in many environments which satisfy requirements of suitable sources of carbon, amino acids and trace elements, where the pH, temperature water activity and osmotic conditions are favourable. Therefore, inoculated into foods during processing can be readily achieved (Farber and Peterkin 1991). Meat, dairy goods, vegetables (Farber and Peterkin 1991), seafood (Dillon and Patel 1992) and products from them are all suitable habitats for *L. monocytogenes* growth and survival. Curiously, an anti-listerial effect has been reported in raw carrots (Beuchat and Brackett 1990; Nguyen-the and Lund 1991; Nguyen-the and Lund 1992). Growth of *L. monocytogenes* has not been reported in grain or cereal products, presumably due to the low water activity of such goods (Lynch and Hobbie, 1988), but pastry products have been contaminated with the organism (Ferron and Michard 1993). Physical structure of a foodstuff will influence spread of a microorganism throughout a given product (Lynch and Hobbie, 1988). For example, a joint of meat is a heterogeneous structure comprising of skin, muscle, connective tissue and bone. These structures present diverse microhabitats for *L. monocytogenes* colonisation, and in bone

constitute a physical barrier to spread. In a study of freshly slaughtered meat, muscle cores were found to be contaminated with *L. monocytogenes* (Johnson *et. al.*, 1990). However, *L. monocytogenes* contamination is a particular problem in foodstuffs that are physically and biochemically homogenous in nature, for example, milk, sausages and paté (Farber and Peterkin, 1991). Listerial contamination in milk is a particular problem as it has not yet been conclusively shown that cell numbers in host (bovine) macrophages is sufficiently reduced by the pasteurisation process (Bunning *et. al.*, 1988; Doyle *et. al.*, 1987). Host phagocytes contain significant amounts of the iron source ferritin for *L. monocytogenes* growth (Kemp *et. al.*, 1993). Increased numbers of process steps in a food production statistically increase risks of microbiological contamination (Doyle *et. al.*, 1988). This is particularly pertinent for *L. monocytogenes* where contact time of as little as 20min. is sufficient for a functional biofilm to be initiated on steel and glass surfaces (Mafu *et. al.*, 1990). Attachment of *L. monocytogenes* to surfaces in flowing systems is significantly enhanced in the presence of an exopolymer-producing bacterium, *Pseudomonas fragi* (Sasahara and Zottola 1993). Formation of active biofilm has considerable implications for the food industry, due to increased cleansing costs (Zottola and Sasahara 1994). In contrast, processing of raw milk into Camembert cheese involves a ripening process where *L. monocytogenes* exploit a microhabitat ≤2mm from the rind of the cheese (Body and Wimpenny 1992). Total colonisation of cheese is possibly prevented by metabolic products, and physio-biochemical conditions in the cheese matrix (Sulzer and Busse 1991). Yoghurt production by *Streptococcus thermophilus* and *Lactococcus bulgaricus* reduce culture pH rapidly which prevents *Listeria* colonisation (Farber and Peterkin 1991). Background microflora in excess of 10^5 cfu.g^{-1} was sufficient to prevent *L. monocytogenes* colonisation in meat held at 7°C (Farber and Peterkin 1991), and this was attributed to the action of Lactobacilli. Food preservation by salting, smoking and freezing have various effects on *L. monocytogenes* growth and survival. Salt preservation is likely to prevent *L. monocytogenes* proliferation in foodstuff when present at > 10%(w/v.) (McClure *et. al.*, 1989). However, survival will

be dependent on foodstuff composition as both betaine peptone and carnitine are accumulated intracellularly and confer tolerance to osmotic stress (Beumer *et. al.*, 1994; Ko *et. al.*, 1994; Amezaga *et. al.*, 1995 and Verheul *et. al.*, 1995). Betaine and carnitine are significant components of plant and animal tissues, respectively (Belitz and Grosch 1986). Smoking of foods will decrease the water activity of a foodstuff to an inhibitory level for *L. monocytogenes* growth, and dependent on smoking method employed, impregnate the outer layers of the food with phenolic compounds which have antimicrobial properties (Lynch and Hobbie 1988; Farber and Peterkin 1991). Storage at 4°C will enrich *L. monocytogenes* numbers (Gray *et. al.*, 1948), but growth can be prevented by freezing. This effectively reduces water activity to levels inhibitory for growth and formation of intracellular ice crystals which may lead to cell burst upon defrosting (Lynch and Hobbie 1988). However, *L. monocytogenes* has been shown to survive freezing in meat (Dickson 1990), possibly due to uptake of cryotolerant substances. such as glycine betaine (Ko *et. al.*, 1994) or location in fatty tissues (Farber and Peterkin 1991). Packaging seems to have little influence on the survival of *L. monocytogenes* in storage (Doyle 1988) and survival has been demonstrated in foods packed under vacuum, to provide anaerobic conditions and under cling-film (Farber and Peterkin 1991).

Given the apparent ubiquity and tenacity of this organism in foodstuffs, it is perhaps surprising that cases of Listeriosis are not frequent. However, the minimum infective dose for healthy humans is estimated to be 1×10^6 -1×10^9 cfu (Farber and Peterkin 1991). Many healthy individuals will consume small quantities of the organism each day without suffering the disease. Indeed, many asymptomatic individuals possess T-cells with reactivity to *Listeria spp.* or immunologically similar bacteria (Munk and Kaufmann 1988) and consequently, T-cell immunity compromised individuals are at increased risk of listeria-mediated infection. Detection of *L. monocytogenes* cells, or nucleic acids in a foodstuff does not necessarily lead to disease following consumption (Hof and Rocourt 1992).

Inhibition of *Listeria monocytogenes* growth due to naturally occurring microbial competition has been observed in milk, cheese, yoghurt and meats (Giraffa *et. al.*, 1995; Sulzer and Busse 1991; Schaak and Marth 1988; Winkowski *et. al.*, 1993). Proteins inhibitory towards *L. monocytogenes* (bacteriocins) have been detected from Lactobacilli (Winkowski *et. al.*, 1993) and *Enterococcus faecium*, commonly isolated from milk (Giraffa *et. al.*, 1995). Presence of bacteriocins inhibitory towards *L. monocytogenes* has been demonstrated in production of fermented sausages (Berry *et. al.*, 1990). In addition, an indigenous bacteriostatic lactoperoxidase system has been reported in milk (Earnshaw and Banks 1989), which has a limited effect on *L. monocytogenes* growth.

Temperature abuse of foodstuffs will undoubtedly have a significant effect on *L. monocytogenes* populations in foods. Prolonged storage at 4°C will enrich for the organism, and salad goods are particularly vulnerable (Farber and Peterkin 1991). Slow rates of heating and cooling during food preparation (or storage) will enable acquisition of thermotolerance, growth and repair following sublethal injury, although numbers will be dependent on the type of foodstuff and composition of the surrounding microbial flora.

Increased process times and steps will, in general, increase risks of *L. monocytogenes* contamination. Prolonged storage and distribution chains, from producer to consumer will, in general, increase the risk *L. monocytogenes* proliferation in a foodstuff.

1.6. Methods of *L. monocytogenes* Detection.

An ideal detection assay for a pathogen such as *L. monocytogenes* should combine rapidity, specificity, and sensitivity, and be indicative of activity in an

environmental sample. Both non-molecular and molecular techniques are currently used for *Listeria* detection and their design and application is discussed below.

1.6.1. Culture-Dependent Techniques

Culture dependent techniques of *L. monocytogenes* detection are designed to indicate the presence of the organism by selective isolation and/or selective enrichment permitting recovery and growth in excess of the surrounding microbial flora.

Historically cold enrichment at 4°C for *L. monocytogenes* exploited the ability of the organism to grow at this temperature (Gray *et. al.*, 1948). However, this may take weeks or even months to occur and its use is therefore somewhat limited (Patel and Beuchat 1995). Enrichment broths have been supplemented with various selective agents to improve their efficacy; for example acriflavine, nalidixic acid and cycloheximide (Donnelly and Baignet 1986; Pine and Gilbert 1988). Often evaluation of these reagents has been performed on a rather empirical basis. However, incorporation of lithium chloride and ferric ammonium citrate into a strictly anaerobic enrichment and repair broth permitted *L. monocytogenes* to compete favourably with background microflora (Mendonca and Knabel 1994).

Incorporation of a repair step in *L. monocytogenes* enumeration significantly improved recovery from clinical samples (Busch and Donnelly 1992). Sublethally injured cells were repaired in a non-selective buffered medium incorporating glucose, yeast extract, pyruvate, Mg^{2+} and Fe^{2+} ions. Repair was complete after 5 h. at 37°C. Following repair, enrichment for *L. monocytogenes* was executed with a selective broth that contained acriflavine, cycloheximide and nalidixic acid for 19 h. at 30°C. This two-step process resulted in significantly increased levels of detection compared to other enrichment broths (Patel and Beuchat 1995). Detection levels were further improved by addition of catalase during the recovery stage (Patel and Beuchat 1995).

Various formulations of selective plating media have incorporated combinations of antibiotics: cycloheximide, moxalactam, nalidixic acid, bactracin, ceftazidine, polymyxin, cefotetan, fosfomycin, colistin and selective agents; acriflavine, glycine anhydride, phenylethanol, lithium chloride, aesculin, potassium tellurite and tween 80 (Farber and Peterkin 1991).

Use of culture dependent methods in isolation has several drawbacks. The methods take considerable time, typically between 24 and 48 h. to achieve colonies that can be tentatively identified as *L. monocytogenes*. Further identification is performed to confirm the presence of *L. monocytogenes* (Kerr *et. al.*, 1990). Selective isolation techniques are not enumerative in nature. Therefore no information can be reliably gained concerning bacterial numbers in an environmental sample. Recovery of viable colonies cannot be guaranteed from sublethally injured populations due to competition from other microbial flora and selective agents in the media used. Colonies that are isolated may show increased resistance to injury, and possibly represent different strains from those isolated subsequently from a patient.

1.6.2. Antibody-mediated detection of *L. monocytogenes*.

Antibody-mediated detection of *Listeria spp.* has been reported with polyclonal antibodies to surface proteins (Ruhland *et. al.*, 1993) and monoclonal antibodies to surface antigens (Kathariou *et. al.*, 1994). Commercial detection methods employ anti-flagella antibodies in fluorescent enzyme linked immunoassays (Parry *et. al.*, 1990; Mattingly *et. al.*, 1988; Kerr *et. al.*, 1990).

Antibody techniques are an advance on culture based methods in that they may be semi-quantitative in nature. A positive reaction is dependent on presence of the appropriate antigen; flagellin production occurs in *Listeria* between 25-30°C (Farber and Peterkin 1991). Enrichment and selective isolation are required for sufficient flagellin production to permit *Listeria* detection. Direct antibody-mediated detection of

listeriae from environmental samples has been successfully achieved from milk only (Donnelly and Baignet 1986). Other detection methods described here require enrichment steps of at least 12 h. before assay; this counters any decrease in assay time compared to plate counts. In addition, the selectivity of anti-*Listeria* antibodies may present a problem. Excepting antibodies against intra invasive protein (Kathariou *et. al.*, 1994), antibody detection methods described here are genus-specific. Monoclonal antibodies directed toward surface proteins of *L. monocytogenes* associated with invasive ability were shown to be strongly reactive with *L. monocytogenes* serotype 4b only, and cross reactivity was reported with some strains of *L. innocua* tested (Kathariou *et. al.*, 1994).

1.6.3. Nucleic acid based detection of *L. monocytogenes.*

L. monocytogenes has been detected with both RNA and DNA directed nucleic acid probes; DNA directed polymerase chain reaction primers and analysis of nucleic acid polymorphisms.

DNA: Identification of *L. monocytogenes* by nucleic acid based techniques initially used DNA probes complementary to a part of the β-haemolysin gene in colony hybridisation studies was observed (Datta *et. al.*, 1987). Hybridisation to *L. monocytogenes* strains was successful, but some cross reactivity was observed with *E. coli* DNA. Successful colony hybridisation systems to *Listeria monocytogenes* DNA targeted the haemolysin A gene (Mengaud *et. al.*, 1988).

RNA: Analysis of RNA with oligonucleotide probes offers potential species specific detection and possibly semi-quantitative information concerning physiological state. This is described further in section 1.8.1. Detection of *L. monocytogenes* by nucleic

acid probes directed to 16S rRNA was initially reported by King *et. al.* (1989) who used a technique that employed a fluorescently labelled capture and detection system.

Polymerase Chain Reaction.

PCR based detection targets areas of the genome which are potentially *L. monocytogenes*-specific. Sequences of interest have been found both in pathogenicity associated and 16S rRNA genes. These are summarised in Table 1.6.5.1. and 1.6.5.2. Detection systems that use PCR primers complementary to sections of the haemolysin gene assume the gene is present only in *L. monocytogenes*. Potential sequences of the haemolysin gene have been identified in non-pathogenic species, *L. ivanovii* and *L. seeligeri* (Farber and Peterkin 1991). Amplification of *Listeria* DNA with *flaA* gene primers is a genus-specific detection method that targets primers complementary to intra-invasive-protein (*iap*) gene target, an area of the genome essential for *L. monocytogenes* pathogenicity (Bubert *et. al.*, 1992; Strachan and Gray 1992). This PCR target tentatively groups *L. monocytogenes* isolates into serogroups. Detection systems have also targeted the delayed hypersensitivity gene (*dth*) and phosholipase gene and *prfA* (Fluit *et. al.*, 1993; Cooray *et. al.*, 1994).

Amplification of *L. monocytogenes* DNA with PCR primers that target 16S rRNA genes exploits sequence differences demonstrated in two variable regions of the gene (Collins *et. al.*, 1991). However, detection of *L. monocytogenes* on the basis of 16S rRNA sequence alone may be in doubt (Czjaka *et. al.*, 1992). A reputedly stable nucleotide difference between *L. monocytogenes* and *L. innocua* has been used in a successful Ligase Chain Reaction (LCR) for *L. monocytogenes* (Wiedmann *et. al.*, 1993).

For PCR assays described detection limits claimed vary between 1 cfu.g^{-1} and >10^{5} cfu.ml^{-1} (Bessesen *et. al.*, 1993; Fluit *et. al.*, 1990). Detection limits < 10^{3} cfu.ml^{-1} have been claimed in instances where PCR assays were performed on either pure cultures, or samples from enrichment cultures (Deneer and Boychuk 1991; Neiderhauser *et. al.*, 1992; Bsat and Batt 1993; Fluit *et. al.*, King *et. al.*, 1989;

Table 1.6.5.1. PCR-based methods of *L. monocytogenes* detection with primers directed to genes involved in pathogenicity.

PCR primer target	Reference	Detection Limit	Comments
haemolysin gene	Powell *et. al.*, (1994)	10^3 cfu	Removal of PCR inhibitory substances from milk samples.
	Bessesen *et. al.*, (1990)	$>10^5$cfu.ml^{-1}	Detects >18ng DNA by PCR; 5ng by nucleic acid hybridisation
	Deneer and Boychuk (1991)	54 cfu.	Samples removed from pre-enrichment broth after 18 hours incubation
	Fitter *et. al.*, (1992)	50-500cfu.ml^{-1}	Detection limit in seeded water samples
	Neiderhauser *et. al.*, (1992)	1cfu.g^{-1}	Detection limit from overnight enrichment cultures
	Blais and Phillippe (1993)	$5x10^4$cfu.ml^{-1}	Detection of cells added to milk, novel concentration procedure.
	Bsat and Batt (1993)	10^2-10^3cfu.ml^{-1}	
	Cooray *et. al.*, (1994)	10^5cfu.ml^{-1}	Incoroprate wahing and filtration steps to remove inhibitory food components.
fla A gene	Gray and Kroll (1995)	N.K.	Genus specific
	Strachan and Gray (1995)	N.K.	Novel fluoresent detector system connected to fibre optic biosensor
iap gene	Bubert *et. al.*, (1992)	N.K.	Isolates grouped due to sequence variability within *L. monocytogenes*
	Neiderhauser *et. al.*, (1992)	N.K.	Successful on primary and secondary enrichments after 32 hours.
dth factor gene	Fluit *et. al.*, (1993)	1cfu.g^{-1} food	Anti-flagellin antibodies concentrate cells after 24 hour enrichment.
prfA gene *plcB* gene	Cooray *et. al.*, (1994)	10^5 cfu.	Cells removed from milk by concentration and wahing steps to rtemove PCR inhibitory substances.

N.K. = not known.

Table 1.6.5.2. PCR detection of *L. monocytogenes* with primers directed to the 16S rRNA gene.

PCR primer target	Author	Detection limit	Comments
16S rRNA gene	King *et. al.*, (1989)	N.K.	Detection on enrichment culture; used novel flurogenic capture and detector system.
	Wang *et. al.*, (1992)	4-40 cfu	Detection limit determied on organisms seeded into milk and cheese.
	McKay *et. al.*, (1993)	100 cells	Observed decrease in colony counts when organism seeded into water, but PCR remained positive,
	Lantz *et. al.*, (1994)	10^4cfu.ml^{-1}	Direct detection from milk by novel biphasic cell separation technique.
	Cano *et. al.*, (1995)	10^3cfu.ml^{-1}	DNA extracted from foodstuffs by novel silica-guanidium isothiocyante protocol.
	Wiedmann *et. al.*, (1993)	10^1cfu.ml^{-1}	Exploits Ligase Chain Reaction for species specific detection. Detection limit determined in pure cultures.

N.K. = not known.

Wiedmann *et. al.*, 1993). However, detection limits after 40 amplification cycles were claimed by Wang *et. al.*, (1992) to be 4-40 colony forming units from foodstuffs seeded with *L. monocytogenes*. In this example, cells were extracted from foodstuffs by filtration and concentration steps and not enumerated prior to lysis. In addition, difficulty was reported in validating this sensitivity in replicates (Wang *et. al.*, 1992). Assessment of foodstuffs for the presence of *L. monocytogenes* by methods excluding PCR indicated that 12-27% of cooked ready-to-eat poultry and poultry products on sale in British supermarkets contained significant numbers of *L. monocytogenes*, up to a maximum of 700 cfu.g^{-1} (Gilbert *et. al.*, 1989; Kerr *et. al.*, 1990).

Improvements in sensitivity may be gained by enhanced PCR product detection, cell concentration or removal of inhibitory components in environmental samples. Detection sensitivity of PCR products was significantly improved from 18ng to 5ng DNA by nucleic acid hybridisation techniques (Bessesen *et. al.*, 1990). Numerous reports which purport to amplify exclusively *L. monocytogenes* DNA do not publish data concerning PCR product confirmation with nucleic acid probing techniques (Cano *et. al.*, 1995; Cooray *et. al.*, 1994; Fitter *et. al.*, 1992; Gray and Kroll 1995; Lantz *et .al.*, 1994; Neiderhauser *et. al.*, 1992 and Powell *et. al.*, 1994). Novel methods of product detection have used fluorochromes in a capture-reporter system (King *et. al.*, 1989) and immobilised fluorochromes on a potentially quantitative biosensor system (Strachan and Gray 1995).

Assays incorporating PCR primers complementary to more than one gene have been reported as 'multiplex-PCR' techniques. Inclusion of PCR primers complementary to appropriate regions of 16S rDNA and listeriolysin O genes permit detection at family, genus and species level in one reaction and permits tentative conclusions to be drawn regarding the microbial composition of a sample (Lawrence and Gilmour 1994). Alternative multiplex PCR assays for *L. monocytogenes* have been reported (Border *et. al.*, 1990; Neiderhauser *et. al.*, 1992; Fluit *et. al.*, 1993; Cooray *et. al.*, 1994).

PCR based detection methods for *L. monocytogenes* are rapid and relatively sensitive. However, direct detection from environmental samples often fails due to

insufficient cell recovery or inhibition of PCR by substances contained in the food (Wang *et. al.*, 1992; Powell *et. al.*, 1994). Successful detection is usually achieved therefore, from cells removed from enrichment broths after incubation steps (Tables 1.6.5.1 and 1.6.5.2.). In those examples detection by PCR is often perceived as confirmation of culture dependent methods (Bessesen *et. al.*, 1990; Cooray *et. al.*, 1994; Fitter *et. al.*, 1992; Neiderhauser *et. al.*, 1992 and Powell *et. al.*, 1994). Detection of *L. monocytogenes* DNA by PCR in a foodstuff does not necessarily imply that the foodstuff constitutes a health risk (Hof and Rocourt 1992).

DNA Polymorphisms.

Listeria monocytogenes may be identified by comparison of nucleic acid band patterns of environmental isolates with those of known laboratory strains. Digestion of DNA with selected restriction enzymes of fragments is termed restriction enzyme length polymorphisms (RFLP). Amplification with primers of a random sequence and analysis of bands produced is termed random amplified polymorphic DNA (RAPD).

Examination of RFLP patterns observed for 176 *L. monocytogenes* strains and 22 non-Listeria strains separated all *L. monocytogenes* strains from other species. The *L. monocytogenes* group could be subdivided into two major clusters which corresponded to flagellar (H) antigen type (Brosch *et. al.*, 1994).

Successful identification of *L. monocytogenes* was attained by RAPD analysis of the intergenic spacer region between 16 and 23S rRNA genes. PCR primers annealed to conserved sequences of 16 and 23S rRNA genes and amplified the variable region between. This has discriminated *L. monocytogenes* from DNA of 28 other prokaryotic species, but considerable care is required to resolve *L. monocytogenes* DNA patterns from *L. welshimeri* (Jensen *et. al.*, 1993).

Identification of *L. monocytogenes* by RFLP and RAPD techniques require isolation of strains from environmental material prior to analysis. Restriction enzyme patterns have been shown to be influenced by the method of DNA extraction

(Waterhouse and Glover 1993). In addition, to derive reliable data, considerable care must be exercised to standardise DNA concentration, reagent concentration and annealing temperature prior to assay (Neiderhauser *et. al.*, 1994; Yu and Pauls 1992). Environmental DNA must be compared against DNA from appropriate laboratory strains and, ideally, the stability of banding patterns should be assessed with respect to culture passages (Neiderhauser *et. al.*, 1992). Interpretation of results can take considerable skill and requires many replicates, increasing assay time. Time taken for RAPD analysis can be reduced by the use of a capillary air thermocycler, decreasing assay time to less than one hour (Black *et. al.*, 1995).

Polymorphic DNA analysis is of considerable value, however, in epidemiological studies of *L. monocytogenes* outbreaks. RAPD and RFLP have been used to successfully trace the source of clinical *L. monocytogenes* isolates to food processing plants (Harvey and Gilmour 1994; Neiderhauser *et. al.*, 1994). RFLP analysis and multilocus enzyme electrophoresis have shown substantial agreement in relationships shown between *L. monocytogenes* strains tested (Harvey and Gilmour 1994).

1.6.4. Direct detection of pathogenic bacteria in foodstuffs.

Direct detection of *L. monocytogenes* in environmental samples is often problematical (Powell *et. al.*, 1992 Wang *et .al.*, 1992;). However, such detection, if possible, would yield valuable data on the presence and possible numbers of the pathogen *in-situ*. Techniques under development attempt to separate bacterial cells from food material prior to nucleic aid extraction, or extract bulk nucleic acids and remove inhibitory components of food material prior to PCR amplification. These methods have predominantly been developed for *L. monocytogenes* detection, and therefore, are of importance here.

Cell Separation. Separation and concentration of bacterial cells from foodstuffs has been achieved successfully by centrifugation and concentration processes. This was reported for *L. monocytogenes* detection from milk and cerebrospinal fluid (Bessesen *et. al.*, 1990). Centrifugation and washing steps aim to detach cells from the food matrix and remove inhibitory substances from environmental material prior to detection (Wang *et. al.*, 1992; Cooray *et. al.*, 1994). Separation and concentration of bacterial cells has been demonstrated for a variety of common food pathogens, including *L. monocytogenes* by membrane filtration. Food materials are maintained in suspension by a stirred cell apparatus, or cross flow filtration to prevent the filter becoming blocked (Bobbitt and Betts 1992). Magnetic separation is perhaps the most exciting method of cell separation and concentration. Briefly, tosyl-activated magnetic microspheres are coated with *Agaricus bisporus* lectins. These purport to demonstrate increased affinity for binding Gram-positive cells. Bacterial cells linked to the lectin coated beads are released using a competitive substrate, fetuin. When evaluated in different foodstuffs, this system removed ≤47% and ≤50% of *L. monocytogenes* cells added to undiluted milk and ground beef, respectively (Payne *et. al.*, 1992). Detection limits were reported to be $\geq 10^3$ cfu.ml^{-1} cells, but improvement in technique has indicated increased detection limits to 10^2 cfu.ml^{-1} in separation and detection of *Brochothrix* spp. (Grant *et. al.*, 1993). However, problems have been reported in cell separation from some foodstuffs and variation in separation efficiency from different bacterial species (Skjerve and Olsvik, 1991; Payne *et. al.*, 1992). In conclusion, separation of *Salmonella* spp. cells from primary enrichment cultures is recommended (Blackburn *et. al.*, 1993). Magnetic bead mediated procedure for concentration of *L. monocytogenes* was reported by Fluit *et. al.*, (1993). Magnetic beads are coated with monoclonal antibodies selective for some *Listeria* spp. Success was only recorded however, in concentration of *Listeria* cells from enrichment cultures (Fluit *et. al.*, 1993).

Effective separation of *L. monocytogenes* from soft cheese has been demonstrated with an aqueous two phase partition system of polyethylene glycol

(PEG) and dextran beads prior to nucleic acid detection by PCR (Lantz *et. al.*, 1994). Washing steps were incorporated into the technique and detection limits are reputedly 10^4cfu.ml^{-1} of cheese homogenate, although other foodstuffs were not tested.

Nucleic acid extraction. Successful detection of *L. monocytogenes* DNA from milk samples by PCR can be achieved by inhibition of indigenous proteinase in the nucleic acid extraction procedure (Powell *et. al.*, 1994). Recognition of food components inhibitory to nucleic acid detection systems has led to development of several novel nucleic acid extraction techniques. Extraction of RNA has been demonstrated from tissue fluids with a novel cationic surfactant, Catrimox™ (Macfarlane and Dahle 1993). This is of potential use in nucleic acid extraction from food samples. DNA suitable for PCR has been extracted from milk by use of solvents, for example toluene (Dickinson *et. al.*, 1995). Detection limits are reported to be ≥ 10^3cfu.ml^{-1} from *L. monocytogenes* cells in a variety of foodstuffs tested and DNA from non-target sources did not interfere with this. Herman *et. al.*, (1995) reported extraction of PCR suitable DNA from neat milk by an alternative solvent extraction procedure to that above. This employed etachimate, a novel polacrylamide polymer to aid recovery of nucleic acid. Other DNA extraction methods employ guanidium isothiocyanate (Cano *et. al.*, 1995) and concentrated (6M) sodium iodide (Makino *et. al.*, 1995) for a variety of cheeses. Detection limits are reportedly ≥10^3cfu.ml^{-1} and 10^3cfu/0.5g food material, respectively.

Listeria specific phage proteins have recently been reported for efficient extraction of DNA, RNA and proteins (Loessner *et. al.*, 1995). The potential specificity and sensitivity is of interest in nucleic acid extraction from *Listeria* cells concentrated from foodstuffs.

1.7. The Value of the Polymerase Chain Reaction in Microbial Ecology.

DNA amplification by Polymerase Chain Reaction has revolutionised detection of microbial DNA from a wide variety of environments (Wright and Wynford-Thomas 1990). Detection can be rapidly achieved often without prior culture steps and specificity controlled. For example, presence of *L. monocytogenes, Listeria spp.* and domain eubacteria has been reported using primers targeted to appropriate 16S rDNA sequences (Lawrence and Gilmour 1994). Detection of several picogrammes of *L. monocytogenes* DNA has been achieved from a mixed fermentation (Bessesen *et. al.*, 1990). Recently, PCR amplification has been used to detect bacteria *in-situ* (Hodson *et. al.*, 1995). The reaction is ostensibly non-quantitative, but delineation of reaction dynamics has led to development of quantitative methods (Sardelli 1993). Competitive PCR quantifies target DNA in conjunction with a known quantity of 'competitor' DNA. Competitor DNA is co-amplified with target DNA and distinguished from it by alteration of length, or inclusion of a restriction endonuclease site along its length (Siebert and Larrick 1992; Gilliand *et. al.*, 1990). The ratio of competitor to target DNA products permits estimation of the environmental template concentration. Alternate methods of quantitative PCR measure accumulation of product over the course of the reaction. PCR product formation has been measured by removing samples at each cycle for gel electrophoresis, relative radioactivity incorporated is measured by autoradiography. This permits the linear portion of the reaction to be determined, and linear regression analysis used to find the initial template concentration (Nakayama *et. al.*, 1992). Real-time measurement of PCR products has been achieved by monitoring ethidium bromide fluorescence in PCR reaction tubes over successive cycles (Higuchi *et. al.*, 1993). This method is however, reliant on adequate mixing of stain and reaction components; and a sensitive and accurate method of image analysis. Incorporation of an internal 'competitive' control and quantitative determination of PCR products has been described by Young *et. al.*, (1993). This reportedly detects $\leq$1000 copies of target DNA and uses a biotinylated

RNA probe in an enzyme linked assay specific to PCR product-probe hybrids. Quantitative PCR methods have been attempted on RNA templates by reverse transcriptase PCR (Wiesner *et. al.*, 1992). This study reinforced the requirement for an appropriate internal standard.

A principal disadvantage of PCR in clinical microbiological studies is that it merely indicates presence of target DNA and does not represent activity, or potential for pathogenesis. Indeed, some *Mycoplasma* spp. may cause neonatal meningitis, but they are usually present as non-pathogenic commensals (Wright and Wynford-Thomas 1990). There are considerable problems concerning the suitability of environmental DNA isolates for PCR. This is particularly relevant when detecting food-borne pathogens, and has confined detection of *L. monocytogenes* by PCR to a confirmatory role, secondary to culture based techniques (Table 1.6.5.1. and 1.6.5.2.). Indeed, the method of DNA extraction has been shown to have an effect on nucleotide sequence (Waterhouse and Glover 1993). Errors may occur in the amplification procedure due to sub-optimal reaction conditions and reading error rate of the Taq polymerase enzyme (Dunning *et. al.*, 1988). This factor, and the possibility of chimera formation (Liesack *et al.*, 1991) require replicate reactions and product confirmation by nucleic acid probing. Estimation of template nucleic acid concentration by PCR is, at present, a lengthy and variable procedure. In addition, little research has been due on quantitative PCR of a selected target from heterogeneous samples. Presence of a mixture of DNA species has been shown to affect PCR detection limits (Fach *et. al.*, 1993). Recent studies have suggested that the predominant amplification product from a mixture of DNA species will be, in part, determined by the length of both template and primer DNA and G:C ratio of respective template DNA species (Suzuki and Giavononni 1996).

1.8. Measurement of Cellular Activity.

Measurement of the activity of a given microorgansim *in-situ* permits conclusions to be drawn regarding not only presence, but also potential for growth and perhaps, pathogenicity of a given organism. Assessments of physiological state have principally been concerned with levels of RNA and cellular function.

1.8.1. RNA Levels.

Advances in nucleic acid technology have led to a surge in available sequence information concerning both 16S, and more recently 23S rRNA (Amann *et. al.*, 1995). Cellular detection, and estimation of activity is possible at family, genus and species level with appropriate use of sequence information available in accessible databases (Lawrence and Gilmour 1994; Amann *et. al.*, 1995).

It is established that bacterial growth rate and cellular RNA concentration are positively correlated in a number of bacterial species (Kjellgaard and Kurland 1963; Rosset *et. al.*, 1966; Gausing 1977; Kerkhof and Ward 1993; Amann *et. al.*, 1990a/b). The exact nature of this correlation with respect to all growth rates and bacterial species is open to discussion. For example, the linearity shown between growth rate and cellular RNA concentration (usually expressed as RNA/DNA ratio) often breaks down at low growth rates (Kramer and Singleton 1992; Kerkhof and Ward 1993). Further definition of this relationship is limited predominantly by technological deficiencies. Rapidly growing *E. coli* cells possibly contain many tens of thousands of ribosomes (Bremer and Dennis 1987). Increases in RNA/DNA ratios in rapidly growing cells effectively increase detection sensitivity if rRNA targeted probes are used.

RNA levels have been measured during cell starvation for a number of bacterial species (Kaplan and Apirion 1975; Davis *et. al.*, 1986; Moyer *et. al.*, 1990; Kramer

and Singleton 1992; Leser *et. al.*, 1995). These studies indicate overproduction of ribosomes at the onset of starvation (Gourse *et. al.*, 1985). Degradation of ribosomal material follows the dissociation of ribosomal particles (Kaplan and Apirion 1975). Ribosomal degradation below a critical number has been linked to cell death (Davis *et. al.*, 1986). Culture conditions prior to starvation appear to have a significant bearing on starvation tolerance, regarding cellular rRNA and ribosome content, and some slow growing species have been shown to actively pool ribosomes in excess of nutritional demand (Kramer and Singleton 1992; Flardh *et. al.*, 1992).

Control of rRNA synthesis during starvation is not yet fully understood. It is proposed that guanosine tetraphosphate (ppGpp) accumulates when excess functional ribosomes exist and inhibits RNA polymerase. This is termed the stringent response (Jinks-Robertson and Nomura 1987). An alternative, passive control mechanism has been proposed by Jensen and Pederson (1990). The mechanism and control of stable RNA synthesis have recently been the subject of comprehensive reviews by Srivastava and Schlessinger (1990) and Wagner (1994).

1.8.2. Quantification of Nucleic acids.

RNA levels have been quantified by a variety of methods. Ribosomes have been enumerated by centrifugal separation (Flardh *et. al.*, 1992); concentration of rRNA has been measured by radioactivity and fluorescent dye technology and ribosomal stability assessed by differential scanning calorimetry to monitor correlation between increased ribosomal stability and increased thermotolerance of *L. monocytogenes* (Stephens and Jones 1992).

Rates of uptake of a radioactively labelled RNA precursor permitted the relationship between rRNA and batch growth phases to be studied (Davis *et. al.*, 1986) Radioactively labelled oligonucleotide probes have been used to quantify rRNA levels in several bacterial species grown under different conditions both *in-situ* (McSweeney

et. al., 1993) and from nucleic acid extracts (Kramer and Singleton 1991; Flardh *et. al.*, 1992). Quantification was by either liquid scintillation counting (Kramer and Singleton 1992; Flardh *et. al.*, 1992) or scanning densitometry of autoradiographs (McSweeney *et. al.*, 1993).

Fluorescent dye technology may be used to quantify RNA and is of increased sensitivity with respect to absorbance and is safer than radio-isotope uptake (Sambrook *et. al.*, 1989). The relationship between nucleic acid concentration and fluorescence due to ethidium bromide intercalation was established by LePecq and Paoletti (1966). Ethidium bromide stain has been successfully used in determination of RNA/DNA ratios in a slow growing marine bacterium (Kerkhof and Ward 1993). Ethidium homodimer offers increased sensitivity compared with ethidium bromide (Markovits *et. al.*, 1979) and this was used to assess nucleic acid levels during cell starvation (Moyer *et. al.*, 1990). Fluorescence in both instances was quantified by fluorometry. Alternative fluorescent nucleic acid stains have recently been developed and offer increased sensitivity compared to ethidium bromide (Green I, II and Hoechst 3334 bisbezimide; Lebaron and Joux 1994).

Oligonucleotide probes may be labelled with fluorescent, antibody, or dioxygenin reporter molecules. These are used to hybridise to specific targets within the bacterial cell under suitable conditions (for review see Amann *et. al.*, 1995). Such methods have been employed to study rRNA levels in a number of bacteria in both laboratory and environmental experiments (Amann *et. al.*, 1990 a/b; Amann *et. al.*, 1992; Hahn *et. al.*, 1993; McSweeney *et. al.*, 1993; Poulsen *et. al.*, 1993; Moller *et. al.*, 1995). Quantitation may be obtained visually with scanning laser confocal microscopy (Caldwell *et. al.*, 1992), or a charge coupled device camera linked to appropriate computer software to permit real-time image analysis of cellular fluorescence levels (Poulsen *et. al.*, 1993; Leser *et. al.*, 1995; Moller *et. al.*, 1995). If suitable samples are available flow cytometery can be performed to discriminate cells on the basis of size and relative fluorescence (Amann *et. al.*, 1995; Lebaron and Joux 1994; Wallner 1995; Muirhead *et. al.*, 1985; Page and Burns 1991).

Analysis of RNA concentration with oligonucleotide probes has permitted study of specific microbial components in mixed fermentations from environments such as activated sewage treatment effluent and rumen microflora (Amann *et. al.*, 1990; Kane *et. al.*, 1993; McSweeney *et. al.*, 1993; Leser *et. al.*, 1995). These techniques have also been used with success to monitor individual components of young, and established biofilms (Poulsen *et. al.*, 1993; Moller *et. al.*, 1995). Studies of this kind are vital in our understanding of microbial function in the environment.

1.8.3. Disadvantages of the RNA approach.

In common with other nucleic acid techniques of microbial analysis, RNA detection relies on a suitable nucleic acid extraction method. Analysis of RNA poses increased degradation risks with respect to DNA due to action of RNase enzymes which exhibit resilience to both thermal and chemical degradation (Blumberg 1979). Degradation may be reduced for example by solubilisation in powerful solvents such as formamide (Chomczynski *et. al.*, 1992).

To obtain a reliable measure of RNA concentration from environmental samples, the extraction efficiency must be assessed. This problem has often been overlooked, but recent studies have addressed nucleic acid extraction efficiency (Kramer and Singleton 1993; Kerkhof and Ward 1993). Extensive calibration and standardisation are required for quantitative RNA detection by nucleic acid hybridisation. Conditions and dynamics for successful quantitative DNA and RNA hybridisation are described by Anderson & Young (1985) and Young & Anderson (1985). Fluorescent analysis of signal by flow cytometry requires a clean liquid sample. Consequently, analysis of foodborne pathogens by flow cytometry directly from samples has only been successfully reported for milk (Donnelly and Baignet 1986). *In-situ* detection of rRNA may be affected by autofluoresence, signal depletion, lack of target nucleic acid, inaccessability of nucleic acid probe binding site and cell

impermeability (Hahn *et. al.*, 1993; Amann *et. al.*, 1995). Methodical study of each problem is leading to improvements in the sensitivity and discriminatory power of nucleic acid probes in a variety of situations.

At low growth rates, the RNA/DNA ratio is significantly decreased and nucleic acid may approach detection limits. This is particularly relevant for slow growing organisms such as *Mycoplasma* spp. and ammonia oxidising bacteria. In environmental samples, it is unlikely that many organisms are growing at maximal growth rates. Colonisation of Camembert cheese by *L. monocytogenes* has been demonstrated but solely in the vicinity of the rind (Robinson and Wimpenny 1992). Presumably, further colonisation from the existent niche is limited by physiochemical composition, toxic metabolites, microbial competition, or a combination of these factors. After initial growth the population remains static, therefore rRNA levels would be greatly reduced indicative of a slowly growing organism, or fail to be detected at all. This does not however, indicate that the organism would not present a health risk if transferred to a suitable environment.

1.8.4. Alternative Measurements of Cellular Activity.

Other measurements of cellular activity record the presence of enzymes required for essential cell functions. These include detection of membrane potential with dyes such as rhodamine 123 (Kaprelyants 1992; Matseyuma 1984) and oxonol (Wilson and Chusel 1985). Presence of esterase enzymes is detected by carboxyfluorescein diacetate (CFDA) which is cleaved intracellularly to form hydrophilic fluorescent products. Alternative indicators of esterase activity are chemchrome B and calcein blue AM (Musgrove and Hedley 1990). Respiratory activity in bacterial cells may be detected by CTC (Rodriguez *et. al.*, 1992). Fluorescence due to these dyes can be observed by fluorescence microscopy and flow cytometry (Porter *et. al.*, 1995). Detection of fluorescence is affected by sample

autofluoresence and signal depletion from environmental material. These methods are not specific to a particular organism so their use is limited to studies of whole microbial populations, or individual microbial components in isolation.

Monitoring electrical conductance from enrichments cultures has been used to detect *Salmonella spp.* from environmental samples (Parmar *et. al.*, 1992). Conductance profiles measured during growth may discriminate groups of bacteria in sample material (Easter and Gibson 1985; Gibson 1987), but the possibility of different bacterial species exhibiting identical conductance profiles cannot be excluded (Easter and Gibson 1985).

1.9. AIMS

These are broadly categorised as follows:

- Design of molecular probes that specifically target *L. monocytogenes* rDNA and rRNA in a PCR based assay.

- Utilisation of such probes to monitor rRNA and DNA quantitatively and obtain tentative RNA/DNA ratios when *L. monocytogenes* is grown under different conditions.

- Assessment of the quantity and quality of nucleic acid released by various techniques to permit representative nucleic acid sampling.

- Verification of nucleic acid probing data, using fluorescent staining methods.

CHAPTER 2.

ROUTINE TECHNIQUES FOR CULTURE OF *Listeria spp.* AND NUCLEIC ACID ANALYSIS.

2.1.1. Strains and Culture Conditions.

Type strains of *Listeria spp.* were obtained from ATCC (12301 Parklawn Drive, Rockville, Maryland, USA), NCTC (Colindale Avenue, London, U.K.) and Clinical isolates were obtained from the Department of Medical Microbiology, Royal Liverpool University Hospital, Liverpool. Strain details are summarised in Table 2.1. Cultures were maintained on Nutrient agar plates (Nutrient broth, $30g.l^{-1}$, pH 7.2; Agar No.2 Lab M, $12g.l^{-1}$) supplemented with 0.5%(v/v) defibrinated horse blood (Tissue Culture Supplies) (NAB plates) to enable observation of haemolysis, with incubation at 25°C. Overnight cultures were grown at 30°C in TSYGB (Tryptone soya broth, $30g.l^{-1}$, Oxoid, 0.5% (w/v) D-Glucose and 0.3%(w/v.) Yeast extract) for routine cell preparations. Strains were stored at -70°C in 50%(v/v) glycerol in TSYGB.

2.1.2. Chemicals and Reagents.

Routine chemicals and reagents used throughout were of Analar grade (or equivalent) and obtained from either BDH, Fisons, Aldrich or Sigma Chemical Corporation.

Table 2.1. Strains and sources of *Listeria* species studied.

Species Name	Serotype	Source	Catalogue Number
L. monocytogenes	n.h.	ATCC	15313
"	1	"	19111
"	2	"	19112
"	3	"	19113
"	4a	"	19114
"	4b	"	19115
"	4c	"	19116
"	4d	"	19117
"	4e	"	19118
"	n.a.	NCTC	11994
L. ivanovii	"	ATCC	19119
"	"	NCTC	11846
L. seeligeri	"	"	1604
"	"	ATCC	35967
L. innocua	"	"	33090
L. grayi	"	NCTC	10815
L. murrayi	"	"	10812
L. monocytogenes	n.a.	RULHMM	836
"	"	"	847
"	"	"	1005
"	"	"	1800
"	"	"	2073
"	"	"	6550
"	"	"	9313
"	"	"	10999
L.innocua	"	"	1909
"	"	"	2393
"	"	"	2551
"	"	"	7234

Key: n.h.= non-haemolytic; n.a.= not available; ATCC = American Type Culture Collection, 12221 Parklawn Drive, Rockville, Maryland, USA.; NCTC = National Collection of Type Cultures, Colindale Ave., London, U.K.; RULHMM = Royal University of Liverpool Hospital Dept. Medical Microbiology, Liverpool, U.K.

2.1.3. DNA Isolation by Caesium Chloride Density Dependant Ultracentrifugation.

Overnight cultures of *Listeria spp.* were harvested by centrifugation at 740g for 20 min. Pellets were washed once with phosphate buffered saline (PBS) pH 7.4 (to 800ml distilled water, 8g NaCl; 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4 the pH adjusted to 7.4 and made up to 1l. with distilled water) and centrifuged for a further 20 min at 740g. Cell pellets were resuspended in 5ml cell suspending buffer (50mM Tris-HCl, pH 8.0; 1mM EDTA; 0.35M sucrose) containing hen egg lysozyme (Sigma Chemical Corp.) to a final concentration of 25mg.ml^{-1} and incubated at 37°C until lysis was observed by an increase in viscosity. Subsequently, 20ml of lysing solution (4.5M guanidium isothiocyanate, EDTA pH 8.0, 25mM sodium citrate 1%(w/v.) N-lauryl sarcosine and 1% (v/v) 2-mercaptoethanol) was added whilst gently swirling the cell suspension. Caesium Chloride was gradually dissolved in lysates to produce solutions of 37%(w/v) glucose equivalent density. Ethidium bromide (400 µl of a 10mg.ml^{-1}solution) was added to each tube and lysates were carefully loaded into ultracentrifuge tubes (Ultracrimp. DuPont). Nucleic acids were centrifuged in a fixed angle rotor (18 h. @ 55,000rpm, 18°C, LKB RP55T rotor). Bands of DNA could be visualised at 260nm on a transilluminator and carefully transferred to Eppendorf tubes with a 19g syringe. Removal of ethidium bromide was achieved by addition of one volume of isoamyl alcohol and gentle mixing. Separation of organic and aqueous phases was achieved by centrifugation at 9440g and the aqueous phase retained. If any traces of ethidium bromide remained in the aqueous phase then further isoamyl alcohol extractions were performed. Samples were dialysed for 48h. at 4°C against two changes of sterile distilled water and one of TE buffer (10mM Tris.HCl pH8.0, 1mM EDTA). Nucleic acids were precipitated by adding 0.1 volumes of 3M sodium acetate pH5.3 and 2 volumes 90% (v/v) ethanol and incubating for at least one hour at -20°C. DNA was pelleted by centrifugation at 9440g for 30 min. Ethanol was removed, and pellets briefly dried before resuspending in 50-200µl of sterile

Hypersolv water. DNA was quantitatively and qualitativley assessed by agarose gel electrophoresis (see below) and U.V. spectroscopy at λ260 and λ280nm.

2.1.4. Agarose gel electrophoresis.

DNA: Agarose gels (0.8-1.2% w/v) were prepared in 1X TAE buffer (2ml 50X solution TAE; 0.5M Tris.HCl, pH 7.6; 0.05M EDTA; 57.1ml glacial acetic acid) and 2μl ethidium bromide ($10mg.ml^{-1}$) added to 100ml molten agarose prior to pouring the gel. Nucleic acid samples (1-15μl) were mixed with 4μl tracker dye (50%(v/v) glycerol, 50%(v/v) TE buffer, 0.05%(w/v) bromophenol blue) prior to loading on to the gel. Nucleic acids were separated by electrophoresis at 90 Vcm^{-1} for 1h. and visualised on a UV transilluminator.

RNA: Denaturing formaldehyde gels were prepared using equipment suitably treated for RNases and dedicated for RNA work.

1X MOPS gel buffer: 1mM Na_2EDTA; 5mM sodium acetate; 20mM MOPS (3-[N-morpholino]propanesulfonic acid), pH7.0.

10X MOPS gel buffer: MOPS 41.9g; sodium acetate, 4.1g; Na_2EDTA, 3.7g Distilled H_2O to 1litre.

14.3X MOPS gel buffer: MOPS 5.93g; sodium acetate, 0.58g; Na_2EDTA, 0.5g; Distilled H_2O to 100ml.

Formamide was deionised for at least 30min over $25gL^{-1}$ Amberlite resin MB1 (BDH) and stored in the dark at -20°C to prevent oxidation. Formaldehyde was filtered through Whatman No.1 paper.

MOPS loading buffer: 500μl deionised formamide; 70μl 14.3X MOPS gel buffer; 180μl filtered formaldehyde.

For a 100ml gel, agarose was dissolved in 6ml 10X MOPS gel buffer and 72ml H_2O by boiling. Upon cooling to below 60°C, 18ml filtered formaldehyde was added.

Samples of RNA (<20μg) were prepared by addition of 16.5μl MOPS loading buffer, and heating to 60°C for 5 min. After addition of 5μl tracker dye, samples were electrophoresed and bands visualised as for DNA.

2.2. Immobilisation of nucleic acids.

2.2.1. Southern transfer of DNA.

DNA samples were transferred from agarose gels to Positive^{TM} (Appligene) nylon membrane by capillary action according to the manufacturers' recommendations: ("Membrane Transfer and Detection Methods", "Hybond^{TM} -N+: Protocols for nucleic acid blotting and hybridisation", Amersham). In all experiments, 0.4M NaOH was used for alkaline transfer of nucleic acids.

2.2.2. Immobilisation of RNA and DNA using a vacuum manifold.

DNA and RNA were applied to positively charged nylon membrane (Positive^{TM} , Appligene) with a Minifold II manifold (Schleicher and Schuell), which had a sample footprint of 6 mm^2. Experimental conditions followed the membrane manufacturers recommendations (see above) and those of Sambrook *et. al.*, (1989).

Prior to RNA immobilisation, the slot blot manifold was soaked overnight in 0.5%(v/v) diethylpyrocarbonate (DEPC) treated water to inhibit RNase activity. Samples of RNA (maximum volume 50μl) were mixed with 3 volumes of a solution containing 70%(v/v) deionised formamide, 24%(v/v) of a 37%(v/v) filtered formaldehyde solution and 6%(v/v) 20x SSC (175.3g NaCl, 88.2g sodium citrate, dissolved in 800ml DEPC distilled water and adjusted to pH7.0 before making up to 1 litre). The samples were then heated at 68°C for 15min, chilled on ice and 2 volumes

of ice cold 20x SSC added prior to storage on ice until required. The samples were applied to the manifold following manufacturers instructions, and the slots rinsed twice with 200µl 20x SSC under vacuum.

DNA samples (maximum volume 50µl) were heated to 95°C, chilled on ice and one volume of ice cold 20X SSC added before storage on ice until required. Subsequent application of DNA samples to the membrane followed the procedure described above for RNA.

Nucleic acids were fixed on to the nylon membrane by air drying for 1h. and heat-fixed at 80°C for one hour. Membranes could be stored, wrapped in aluminium foil at 4°C, prior to further treatments.

2.2.3. Oligonucleotide probe labelling.

Oligonucleotide probes were commercially synthesised (Dept Biochemistry, Kings College, London or Unilever Research, Colworth , Bedford), quantified by U.V. spectroscopy (at λ260 and λ280nm) and diluted to a final concentration of 10pM.µl^{-1}. Probes were end-labelled with γ-^{32}P-dATP (ICN Supplies). Briefly, 10pM oligonucleotide, 1µl 10X kinase buffer (Tris.HCl, 50mM; $MgCl_2$, 10mM; EDTA, 0.1mM; dithriothreitol, 5mM; spermidine, 0.1mM; pH8.2), 6µl sterile hypersolv water, 1µl T4 phage polynucleotide kinase, (Boerhinger Mannheim 174645) and 1µl γ -^{32}P-dATP (370KBq) were mixed and incubated at 37°C for one hour. The labelling reaction was stopped by heating at 75°C for 5min. Probe labelling efficiency was assessed as follows. Aliquots (1µl of a solution containing 10% (v/v) reaction mix and sterile hypersolv water) were spotted on to squares (1 cm approximately) of DE81 paper (Whatman, 3658-023). Upon drying, 50% of the membrane samples were rinsed in 300ml ice cold 0.5M Na_2HPO_4 for 2 min. The membranes were transferred to a

beaker containing a fresh solution of 0.5M Na_2HPO_4 and the process repeated twice more before briefly rinsing the filter in 70%(v/v) ethanol and air drying. Subsequently, the membranes were inserted in scintillation counter vials and radioactivity quantified by counting Cerenkov radiation using the ^3H channel of a scintillation counter (Beckman LS 1801). Comparison of the radioactivity present on the unwashed and washed filters (which retained only labelled oligonucleotide probe) revealed the percentage labelling efficiency. Labelling efficiency in excess of 20% was deemed satisfactory.

2.2.4. Oligonucleotide probing solutions.

10% Blocking Reagent Solution: Maleic acid (0.1M) and NaCl (0.15M) were dissolved in 500ml DEPC treated water and adjusted to pH 8.0 by the addition of NaOH. Blocking reagent (50g/500ml, Boerhinger Mannheim, 1096176) was dissolved in this solution prior to autoclaving. The solution was stored at 4°C or -20°C prior to use at 2%(w/v) in prehybridisation solution.

Prehyridisation Solution: Blocking reagent (2% w/v); SSPE, 5x (Briefly, to 800ml distilled H_2O was added NaCl, 45.83g; $NaH_2PO_4.H_20$, 27.6g; EDTA, 7.4g; adjusted to pH 7.4 and made up to 1L); deionised formamide, 20%(v/v); SDS, 0.02%(w/v); N-laurylsarcosine, 0.1%(w/v) were prepared in sterile DEPC treated water. Sufficient solution was used to cover the membranes and prehybridisation was for a minimum of one hour at room temperature.

Hybridisation/Wash solution: SSPE, 5x; deionised formamide, 20% (v/v); SDS, 0.02% (w/v); N-laurlysarcosine, 0.1% (w/v) were prepared in sterile DEPC-treated water. Blocking reagent was not included in hybridisation or wash solutions as this may inhibit probe annealing, particularly where probes are less than 100 nucleotides in length, (Sambrook *et. al.*, 1989). Hybridisations were carried out at the specific

annealing temperature (determined experimentally) overnight and washed at room temperature for 5min.

2.2.5. Preparation of solutions and glassware for RNA work.

Solutions were treated with 0.5%(v/v) DEPC (Aldrich Chemical Corp.), an inhibitor of RNase activity (Blumberg, 1987). Where compounds were unsuitable for this treatment (those containing Tris buffer, for example) they were prepared in autoclaved DEPC treated water. Glassware, soaked overnight in DEPC water was autoclaved and dried at 65°C. Equipment unsuitable for autoclaving was soaked overnight in DEPC water. All work was performed whilst wearing disposable gloves.

CHAPTER 3.

OPTIMISATION OF DIAGNOSTIC PCR PRIMERS AND OLIGONUCEOTIDE PROBES FOR *Listeria monocytogenes* DETECTION.

3.1. AIMS.

- To design PCR primers and oligonucleotide probes for diagnostic detection of *L. monocytogenes* 16S rDNA and rRNA.

- To optimise nucleic acid hybridisation conditions to permit quantitative oligonucleotide probing of both rDNA and rRNA.

- To extract both DNA and rRNA from foodstuffs in a form suitable for PCR amplification and quantitative oligonucleotide probing.

3.2. MATERIALS AND METHODS.

3.2.1. Design of PCR primers and oligonucleotide probes.

Deposited sequences of 16S rRNA for members of the genus *Listeria* (EMBL database accession numbers X56148-X56154, Collins *et. al.*, 1991) were screened for suitable diagnostic oligonucleotides. Areas of interest were found in the V2 and V9 regions (143-220bp. and 1238-1297bp. *E. coli* 16S rRNA sequence numbering; Edwards *et. al.*, 1989) where mismatches existed between *L. monocytogenes* and *L. innocua*. These species exhibit the most sequence similarity of the genus *Listeria* (Collins *et. al.*, 1990; Czjaka *et. al.*, 1993). Possible oligonucleotides were screened using the CHECKPROBE package provided by Ribosomal Database Project (RDP; Maidak *et. al.*, 1994) for their diagnostic value and possible artefact formation, for example primer-dimerisation. In addition, oligonucleotide probes were designed to be diagnostic for *L. ivanovii* and *L. seeligeri*. The sequences thus selected are shown in Table 3.2.1. The position of these sequences on the secondary structure model of 16S

rRNA by Woese *et. al.*, (1983) is shown as Fig. 3.2.1. Oligonucleotides were commercially synthesised and cleaved from support columns at either the Department of Medical Microbiology, Kings College, London or Unilever Research, Colworth, Bedford. Concentrations were verified upon arrival by U.V. spectroscopy. Prior to practical evaluation, the theoretical reversible melting temperature (T_m) of the oligonucleotides was calculated (Johnson, 1991).

3.2.2. Determination of irreversible melting temperature of target:probe DNA duplex .(T_i).

The general procedure followed was that of Sambrook *et. al.*, (1989) and Wallace *et. al.*, (1979).

Target nucleic acids (approximately 100ng per slot) were applied to a charged nylon membrane (Positive™, Appligene) using a vacuum filtration manifold as described previously. After air drying and baking, the filters were cut to a size slightly larger than the sample footprint before prehyridisation for 1h. in the solution described above. Subsequently, the filters were rinsed in a small volume of wash solution and submerged in 10ml volume of hybridisation solution containing 10pmol end-labelled oligonucleotide. Filters were hybridised at room temperature overnight without agitation. The filters were then washed in 10ml ice cold wash solution for 5 min. and the process repeated with fresh ice cold wash solution until all non-specifically bound probe was removed. This was checked with a hand-held geiger counter. The water bath was set at -25°T_m (reversible melting temperature of the target:probe duplex estimated according to Johnson *et. al.*, (1991)). Aliquots (3mls) of wash solution were put into each of 20 test tubes, of which 5 were placed in the bath to equilibrate. Upon use, these were replaced to permit further tubes to be equilibrated.

Table 3.2.1. Sequences of oligonucleotide probes and PCR primers used in this work.

Designation	Eubacterial (U) or Listeria specific (L) 16S rRNA target.	Sequence 5' → 3'	Approximate annealing position (bp) after Edwards *et. al.* (1989)
pA	U	AGA GTT TGA TCC TGG CTC AG	8-28
pB	U	TAA CAC ATG CAA GTC GAA CG	50-70
pD'	U	GTA TTA CCG CGG CTG CTG	536-518
pE'	U	CCG TCA ATT CCT TTG AGT TT	928-908
pF	U	CAT GGC TGT CGT CAG CTC GT	1053-1073
pG'	U	ACG GGC GGT GTG TAC	1407-1392
pH'	U	AAG GAG GTG ATC CAG CCG CA	1542-1522
MV2	L	GCT AAT ACC GAA TGA TAA AG	167-186
MV9R	L	AGA ATA GTT TTA TGG GAT	1304-1287
MV9P	L	GCG AGG TGG AGC TA	1273-1286
MV9RP2	L	ATA GTT TTA TGG GAT TAG CTC	1301-1281
IP	L	GAT GAC ATG CGT CAC TAC	201-185
SP	L	GTG ACA TGC GTC ACT CC	201-184

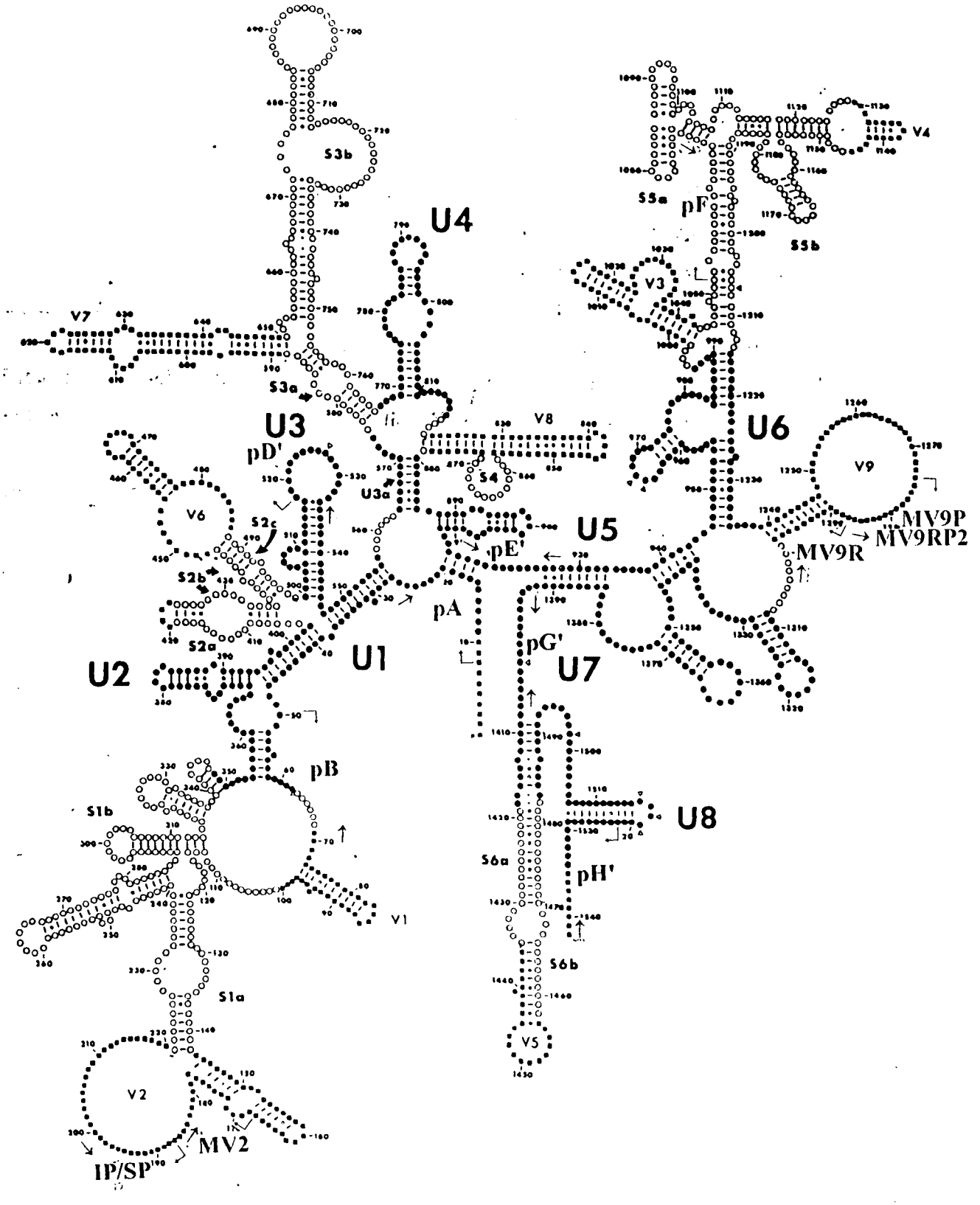


Figure 3.2.1. Eubacterial (*E. coli*) 16S rRNA secondary structure model proposed by Woese *et. al.*, (1983) showing regions where oligonucleotide probes and PCR primers used in this work are targeted. Universal: U; Semi-conserved: S and variable: V regions of SSU rRNAs.

A thermometer was placed in an identical tube containing one ml wash solution and placed in a waterbath. A volume (approximately 100ml) of wash solution was kept at room temperature. When the tubes containing wash solution in the water bath had equilibrated, 1ml was transferred to each testube containing a filter. After 5min incubation, the solution was removed and the filters rinsed with 1ml wash solution held at room temperature. The incubation and rinsing wash solutions were combined in disposable scintillation counter tubes. The process was repeated at 3°C intervals to T_m +30°C, or until most radioactivity had been eluted, and an end-point incubation determined in a boiling water bath. Radioactivity was quantified by counting Cerenkov radiation using the ^3H channel of a scintillation counter (Beckman LS 1801). The results were integrated to produce a sigmoid graph of percentage ^{32}P eluted against temperature, the value at which 50% was eluted corresponding to the Ti. These experiments were performed in triplicate series.

3.2.3. PCR reaction conditions.

For routine amplifications with "universal" eubacterial primers (see Table 3.2.1.; Edwards, *et. al.*, 1989), 500 µl reaction mix was prepared comprising the following: 75 µl, 10 mM $MgCl_2$; 50 µl, 670 mM Tris-HCl pH 8.0; 100 pM each, of forward and reverse primer; 100 mM of deoxynucleotide mix (Pharmacia Biotech.), 1µl 10%(w/v.) bovine serum albumin; 340 µl sterile Hypersolv^{TM} water (BDH 15273) and 5U Amplitaq, DNA polymerase (Perkin Elmer Cetus Corp.). A 0.5-4µl aliquot of template had 96µl of the above reaction mix added to it in a thin-walled reaction tube and was then overlaid with mineral oil to prevent evaporation during amplification.

The reaction parameters were 26 cycles consisting of: 94°C, one min; 55°C, two mins; 72°C two min; after 26 cycles a final elongation step of 20 min at 72°C. Reaction tubes were held at 0°C upon completion.

Amplifications with *Listeria* specific primer pair MV2 and MV9R employed "hot start" PCR (Chou, *et. al.*, 1992). Briefly, into each reaction tube was placed 66 µl of a mixture containing 100 pM of each primer; 100 mM of deoxynucleotide mix; 75 µl 10 mM $MgCl_2$; 50 µl 670 mM Tris-HCl pH 8.0; 200 µl sterile Hypersolv™ water and 1 µl 10% (w/v) bovine serum albumin. This was overlaid with mineral oil and heated in the thermocycler to 75°C. Upon reaching this temperature, 33 µl of a mixture comprising: 25 µl 670 mM Tris-HCl pH 8.0, 5 U Amplitaq DNA polymerase (Perkin Elmer-Cetus Corp.), template DNA (0.5-4 µl) and 140 µl Hypersolv™ water was pipeted into the reaction tubes through the mineral oil layer, and the reagents mixed. Subsequently, reaction parameters followed that of standard PCR reactions as described above except that the annealing temperatures were varied during the course of experiments.

PCR with potentially species-specific primer pair MV2 and MV9R (see Table 3.2.1.) was often performed in templates produced by amplification with "universal" PCR primers pA and pH' (Table 3.2.1.). Products were suitably diluted (1-2.5% (v/v) in Hypersolv water) to prevent interference due to primers from the initial PCR reaction. Primers used in the second amplification annealed to sites internal to those of the primary reaction, and this is termed "nested" PCR. This increased the sensitivity of diagnostic primers for detection of template DNA from original samples.

3.2.4. Evaluation of diagnostic oligonucleotide probes.

Amplifications were performed with extracts of DNA obtained as described previously from the culture collection of *Listeria spp.* (see Table 2.1.). DNA amplification by PCR with universal primers pA and pH' under conditions described above produced 1.5kb fragments which were separated by agarose gel electrophoresis. Fragments were transferred to a nylon membrane with 0.4M NaOH which was hybridised overnight in standard solutions with 10pM γ^{32} P-dATP labelled

oligonucleotides MV9P or MV9RP2 at hybridisation temperatures ranging from 40°C to 60°C. Filters were washed at room temperature for 5 min, double-wrapped in cling film and X-ray film (Fuji-film X-o-graphic) exposed for both 2 and 7 days at -70°C.

Sensitivity of probes pA, MV9P and MV9RP2 were evaluated as follows. Dilution series of *L. monocytogenes* DNA of a known concentration were applied and fixed to nylon filters using a vacuum manifold as described earlier. The filters were probed with 10pmol. of either radiolabelled pA, MV9P or MV9RP2 of a known labelling efficiency; hybridisations were carried out at 40°C overnight. The filters were washed for 5min. at room temperature and the X-ray film exposed as described for previous experiments. The limit of detection of each probe could be compared by both visual examination and scanning densitometry.

3.2.5. Evaluation of diagnostic PCR primers.

Oligonucleotide PCR primers were evaluated for detection of *L. monocytogenes* DNA. Amplifications were done with DNA extracts from the *Listeria spp.* culture collection (Table 2.1). PCR amplification with universal primers pA and pH' produced 1.5 kb fragments. These were diluted as described above in Hypersolv water and 26 cycles of hot start PCR performed with primer pair MV2/MV9R, annealing temperatures ranging from 45°C to 55°C. The expected 1137bp product was separated by agarose gel electrophoresis. Fragments were transferred to a nylon membrane by alkaline transfer and the fragment identity confirmed by oligonucleotide probing with 10pmol. radio-labelled probe MV9RP2 (Table 3.2.1.); hybridisation was carried out 40°C overnight. The filters were washed at room temperature for 5min. and exposed to autoradiographs performed at -70°C for 2 and 7 days.

3.2.6. Quantitative oligonucleotide probing of nucleic acids.

Titration series of either *E. coli* 16 and 23S rRNA (0.4μg.μl^{-1}, Boerhinger Mannheim 206938), *L. monocytogenes* RNA or DNA of known concentrations were applied to a nylon membrane using a slot blot manifold as described in section 2.2.2. A maximum quantity of 24-36μg nucleic acid was applied to saturate the filter (upper binding capacity of Positive™ 400-600μg.cm^{-2}; Hiorns, (1993); unknown for Appligene Hybond N^{+}). Filters were probed at either 25 or 40°C overnight with either 10pmoles γ^{32} P-dATP labelled probes MV9RP2, pE' or pA before washing at room temperature, and X-ray film exposed by autoradiography for 2 and 7 days.

Autoradiograph signal intensity was quantified with Molecular Dynamics Image Quant version 3.2 software running on a Personal Densitometer. Signal (pixel) intensity above background and signal volume above background were transferred to Microsoft Excel version 3.0 software running in Microsoft Windows version 3.11 where further data analysis was performed.

3.2.7. *In-situ* oligonucleotide probing of *L. monocytogenes* rRNA.

Overnight mid-exponential cultures of *L. monocytogenes* (approximately 1 x 10^9 cfu ml^{-1}) were harvested by centrifugation at 740g for 10min. Cells were subsequently washed in PBS and 1ml aliquots resuspended in 0.25ml PBS and either 0.75ml 70% (v/v) ethanol or 0.75ml paraformaldehyde fixing solution (briefly, 2g paraformaldehyde was dissolved in a solution at 60°C containing 5ml 10X PBS; 100μ l 10M NaOH; and 44.5ml sterile hypersolv water. Upon cooling to room temperature, the solution was adjusted to pH 7.2, membrane filtered (0.45μm), stored at 4°C until required) and incubated overnight at 4°C. Cells were pelleted, resuspended in 1ml PBS and approximately 1 x 10^5 cfu.ml^{-1} transferred to fresh Eppendorf tubes. These were pelleted by centrifugation at 740g for 10min. and resuspended in 38μl

hybridisation solution (200µl, 4.5M NaCl; 100µl, 200mM Tris-HCl pH 7.2; 10µl, 10% (w/v) SDS; sterile Hypersolv water and deionised formamide added to produce solutions containing 20, 30, 40 or 50% (v/v) formamide in a total volume of 1010µl). To each tube was added 100ng 5'-fluorescein isothiocyanate (FITC) labelled MV9RP2 (Genosys UK, 162A Cambridge Science Park, Milton Rd., Cambridge, CB4 4GH). Positive and negative controls used throughout were 5' FITC labelled EUB338 (a domain eubacteria rRNA complementary probe) and EUB338' (a non-complementary probe) respectively (Amman *et. al.*, 1990). An additional negative control in the absence of oligonucleotide probe was included to evaluate cell autofluoresence. All samples were hybridised at 37°C for different lengths of time (1 to 24 h.). Upon hybridisation, cells were pelleted by centrifugation at 740g for 10 min and washed once in 10 µl Hypersolv water before resuspending in 10 µl Hypersolv water. Aliquots (5µl) of each sample were pippeted on to microscope slides and coverslips surrounded with a small amount of clear nail varnish to prevent sample dehydration. Slides were examined on a Zeiss Axioplan epifluorescence microscope and recorded photographically (Kodak Ektachrome film).

3.2.8. Extraction of nucleic acids from milk.

To 1ml of 10% (v/v) semi-skimmed UHT milk solution in Hypersolv water was added aliquots containing either 10^9, 10^6 or 10^3 cfu. *L. monocytogenes* cells and thoroughly mixed. Cells were lysed and nucleic acids extracted following either the protocol of Dickinson *et. al.*, (1995) or Catrimox-14™ cationic surfactant (Macfarlane and Dahle, 1993). After cell lysis with Catrimox-14™ the RNA pellet was dissolved in 0.5ml solution consisting of 4M guanidium isothiocyanate, 200mM sodium acetate, pH4.0 and nucleic acids extracted by addition of 1 volume phenol:chloroform:isoamylalcohol (25:24:1) pH 8.0. Preparations were centrifuged for 5min at 9440g, and aqueous phases transferred to fresh ependorf tubes. Nucleic

acids were precipitated at -20°C for one hour by the addition of 0.1 volume 4.5M sodium acetate pH5.3 and 1 volume absolute ethanol. Following centrifugation for 30min. at 9440g, the pellet was briefly washed in 70% (v/v) ethanol, dried and resuspended in 50µl DEPC treated Hypersolv water. Separation of nucleic acids was achieved by 1% (w/v) agarose gel electrophoresis and visualised on a UV-transilluminator.

The nucleic acid extracts obtained were incubated with DNase-free RNase (Boeringer Mannheim 1119915) added (3-15 U μl^{-1}) for 5 h. at 37°C. Remaining DNA was extracted with phenol:chloroform:isoamylalcohol (25:24:1) and precipitated overnight at -20°C in 0.1 volume 4.5M sodium acetate pH 5.3 and 1 volume absolute ethanol. DNA was pelleted by centrifugation for 30 min at 9440g and resuspended in 50 µl sterile hypersolv water. Amplification of this DNA by PCR was achieved using universal eubacterial primers pA/pH' under standard reaction conditions as described previously. Positive controls contained caesium chloride centrifugation extracted *L. monocytogenes* ATCC 19111 DNA. Products were separated by agarose gel electrophoresis and nucleic acids transferred to a nylon membrane by alkaline transfer. Membranes were hybridised at T_i overnight with 10pmoles γ-^{32}P-dATP labelled oligonucleotide probe MV9RP2 and washed at room temperature before exposing X-ray film by autoradiography for 2 and 7 days.

3.2.9. Determination of 16S rRNA gene copy number.

The *L. monocytogenes* 16S rDNA sequence (EMBL No. 56153) was analysed for restriction endonuclease sites. Restriction enzymes were chosen on the basis of this data to cut *L. monocytogenes* DNA outside the 16S rDNA gene. Other selection criteria were that star activity (restriction activity at related, but not specific sequences; Boerhinger Mannheim) should be minimised, and that there was a high

probability of DNA outside the 16S rRNA gene being cut more than once to yield the maximum fragment information possible.

To aliquots (8µl) of 8µl *L. monocytogenes* ATCC 19111 DNA (500ng.µl^{-1}) were added 1µl of either *BamHI, ClaI, EcoRV, HaeII, HindIII, PvuII, StuI* (Boerhinger Mannheim) or *MscI* (New England Biolabs) and 1µl of appropriate endonuclease buffer to individual tubes. Tubes were incubated at 37°C for 3h. and reactions stopped by incubation for 5min. at 75°C. After digestion, 4µl aliquots of gel loading buffer were added to each reaction tube. Fragments were separated by 2% (w/v). agarose gel electrophoresis for 2h. at 75V.cm^{-1} and visualised on an U.V. transilluminator. Alkaline transfer of DNA fragments to Positive™ membrane was performed and nucleic acid fragments fixed to the membrane by the procedures described above. Filters were incubated for one hour at room temperature in pre-hybridisation solution and hybridised overnight at 40°C in the presence of 10pmol. γ ^{32}P-ATP end-labelled universal oligonucleotide pE'. The filter was rinsed in wash solution for 5min. at room temperature, double wrapped in cling film and X-ray film exposed by autoradiography at -70°C for 2 and 7 days before development and analysis.

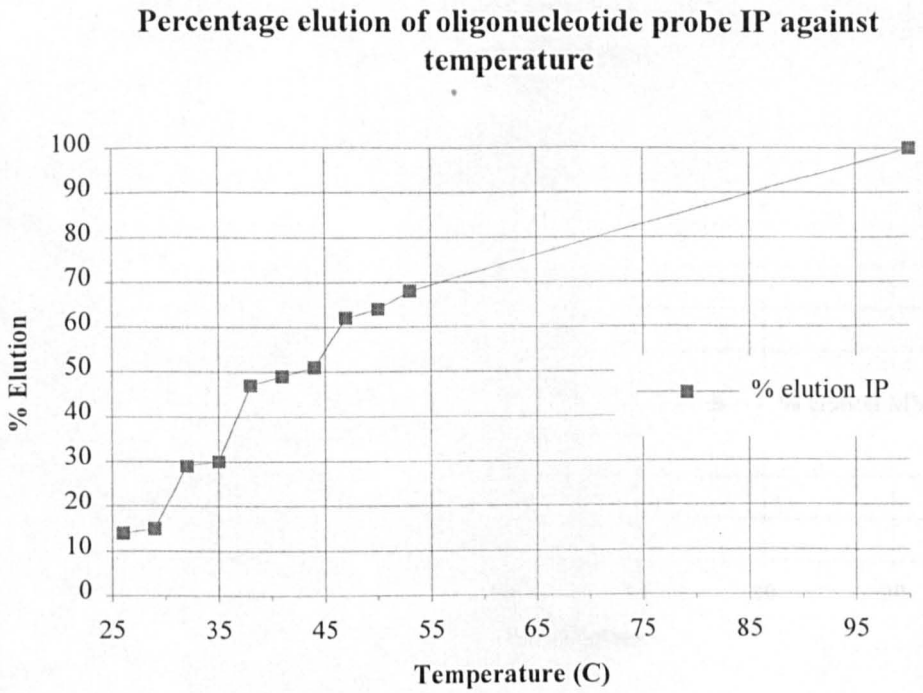
3.3. RESULTS.

3.3.1. Estimation of T_i values for oligonucleotide probes.

The percentage elution of radiolabelled oligonucleotides IP, MV9P, MV9RP2 and SP due to increasing temperature was recorded. These data are represented in Figures 3.3.1a-d. Values pertaining to T_i (irreversible melting temperature, corresponding to 50% elution of radio labelled oligonucleotide) were determined at 41 °, 44°, 42°, and 46°C for oligonucleotides IP, MV9P, MV9RP2 and SP respectively. The diagnostic value of oligonucleotide MV9RP2 was examined by probing PCR products derived from DNA preparations of 20 *Listeria* strains, in hybridisation reactions carried out at 40°, 45°, 55° and 60°C. This is described below in section 3.3.3.

3.3.2. Evaluation of diagnostic PCR primers.

The PCR primers MV2 and MV9R were used to amplify DNA from 20 strains of *Listeria spp.*, with annealing at either 45°, 50°, 53°, 54°, 55° or 56°C. Results are summarised in Table 3.3.1. In general, increasing annealing temperature increased the species-specificity with these primers. At an annealing temperature of 45°C, only two of the DNA templates derived from 20 strains of *Listeria spp.* did not amplify (*L. grayi*, 10815; *L. murrayi*, 10812), whilst annealing at 56°C PCR products from 4 templates (*L. monocytogenes*, 19111, 19113 and 9313; *L. innocua* 33090) were observed. However, the primers MV2/MV9R were not diagnostic for *L. monocytogenes* at this annealing temperature as some *L. innocua* strains were



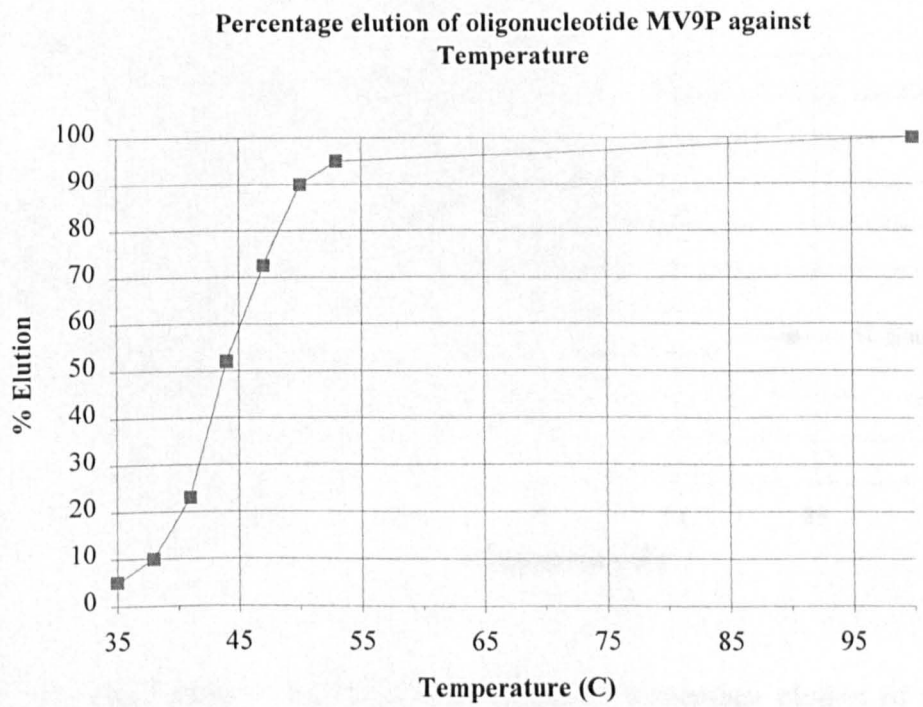
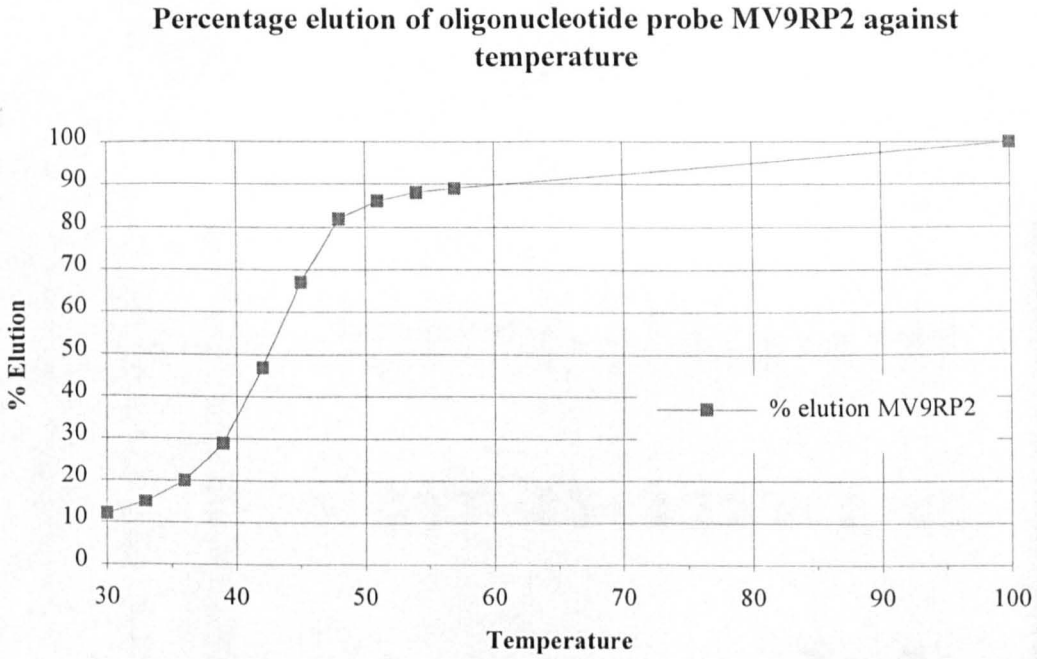


Figure 3.3.1a (upper) 3.3.1b (lower) Graphs of percentage of γ^{32}P-ATP labelled oligonucleotide eluted against temperature. Traces are means of triplicates.



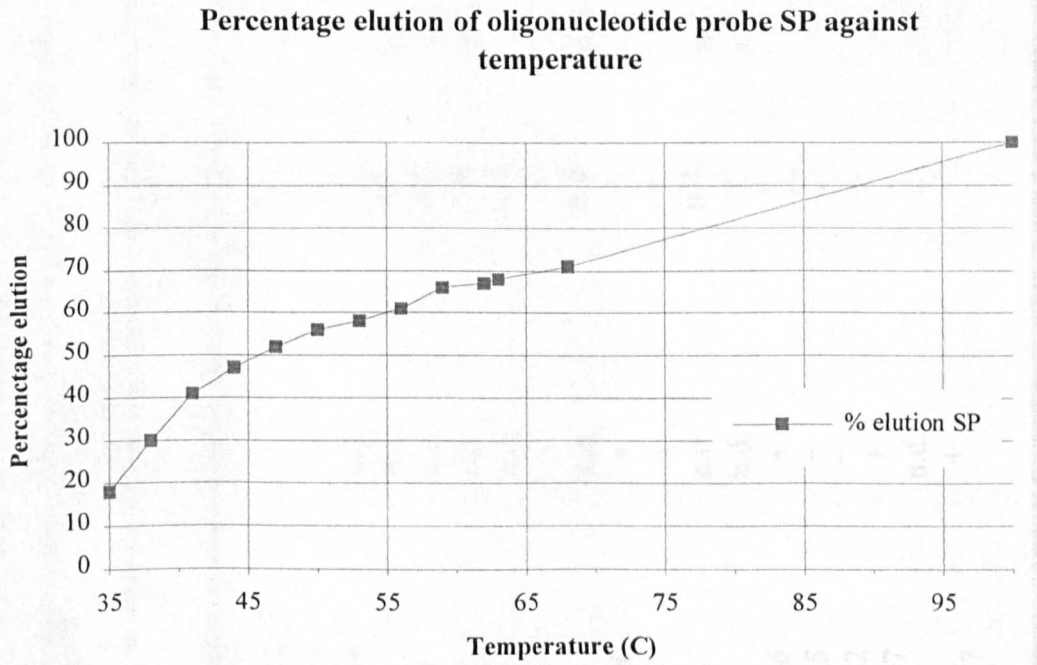


Figure 3.3.1c (upper), 3.3.1d (lower) Graph of percentage elution of radio-labelled oligonucleotide probes MV9RP2 and SP against temperature (°C). Traces are means of triplicates.

Table 3.3.1. Effect of increasing annealing temperature on PCR product formation with primers MV2/MV9R.

Species	Strain No.	Annealing temperature (°C)					
		45	50	53	54	55	56
L. monocytogenes	15313	+	+	+	+	+	−
""	19111	+	+	+	+	+	+
""	19113	+	−	+	+	+	+
""	19114	n.d.	+	+	+	+	n.d.
""	836	n.d.	n.d.	n.d.	+	n.d.	n.d.
""	1005	n.d.	n.d.	n.d.	+	n.d.	n.d.
""	1800	n.d.	n.d.	n.d.	+	n.d.	n.d.
""	2073	n.d.	n.d.	+	+	−	n.d.
""	6550	+	+	+	+	n.d.	n.d.
""	9313	n.d.	n.d	n.d.	+	n.d.	+
L. innocua	33090	+	+	+	+	+	+
""	1909	+	+	+	−	n.d.	−
""	2393	n.d.	n.d.	n.d.	−	n.d.	n.d.
""	2551	n.d.	+	n.d.	−	n.d.	n.d.
L. ivanovii	11846	+	−	−	−	n.d.	n.d.
L. grayi	10815	−	−	−	n.d.	−	−
L. murrayi	10812	−	+	−	−	n.d.	n.d.
L. seeligeri	35967	+	−	+	−	−	−
L. seeligeri	1604	n.d.	−	−	n.d.	n.d.	n.d.
L. welshimeri	15897	+		−		n.d.	n.d.

N.B. + = Product band observed; = no product band observed; n.d.= not determined

amplified (33090) and *L. monocytogenes* 15313 was not amplified.

In Figs. 3.3.2. a-f, a double product band of approximately 660bp size is evident. The cause of this band was investigated by conducting a hot-start PCR assay incorporating a fraction of either radio-labelled forward primer MV2 or similarly labelled MV9R. PCR parameters comprised 26 cycles of: 94°C 1min; 54°C 1min; and 72°C 2min. The fragments produced were separated as above and transferred to a nylon membrane. The membrane was sandwiched against two autoradiograph films and these exposed for either 24h. or 48h. at -70°C. The developed autoradiographs (Fig. 3.3.2.g) shows incorporation of both the forward and reverse primers into both product bands. This indicates the presence of both primers in the product sequences. DNA fragment amplification with PCR primers MV2/MV9R annealing at 54°C were hybridised overnight at 45°C with labelled probes MV9P and MV9RP2. The autoradiographs are shown in Fig 3.3.2.h. Probe MV9P appeared less sensitive than probe MV9RP2 and annealed to the 1137bp fragment only.

Titration of template DNA prior to PCR amplification with primer pair MV2/MV9R and hybridisation of the products with oligonucleotide probe MV9RP2 showed that less than 30pg *L. monocytogenes* template DNA could be detected by this method (Fig. 3.3.2.i).

3.3.3. Evaluation of diagnostic oligonucleotide probes for *L. monocytogenes.*

The oligonucleotide probe designed to be *L. monocytogenes* specific was tested at different hybridisation temperatures against PCR fragments amplified from different *Listeria spp.* DNA with primers pA/pH'. The fragments were separated by agarose gel electrophoresis and transferred to nylon filters as described earlier. These were hybridised overnight at either 40, 45, 55 or 60°C in the presence of 10pmol. radio-

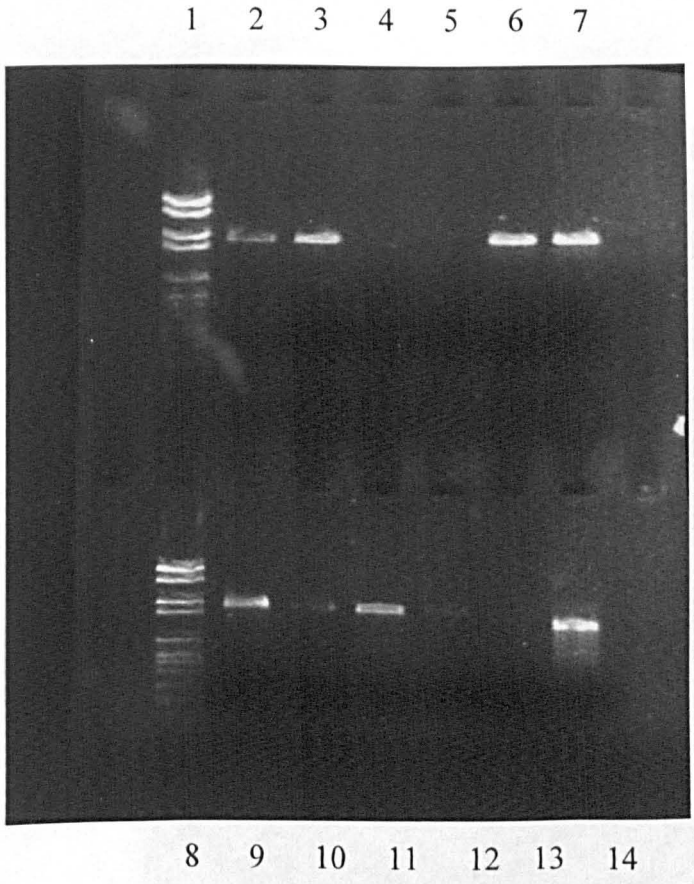


Figure 3.3.2.a. PCR amplification with primers MV2/MV9R annealing at 45°C. Lanes 1 & 8 DNA molecular weight marker VI (Size range: 0.15-2.1Kbp.; Boehringer Mannheim). Template DNA from *L. innocua* 33090 (lane 2); *L. monocytogenes* 19111, (lane 3); *L. murrayi* 10812, (lane 4); *L. grayi* 10815 (5); *L. monocytogenes* 19113 (6); *L. monocytogenes* 6550 (7); *L. monocytogenes* 15313 (9); *L. welshimeri* 15897, (10); *L. seeligeri* 35967 (11); *L. ivanovii* (12); negative control, (13); *L. innocua* 1909 (14).

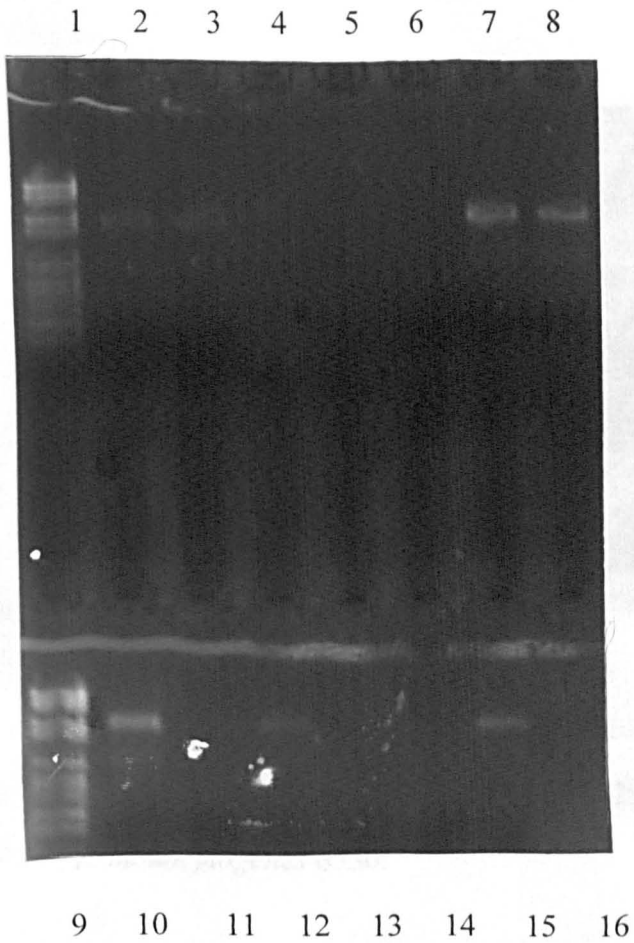


Figure 3.3.2.b. PCR amplification with primers MV2/MV9R annealing at 50°C. Template DNA as follows: Lanes 2-8 respectively, *L. innocua* 33090; *L. monocytogenes* 19111; *L. murrayi* 10812; *L. grayi* 10815; *L. monocytogenes* 19113; *L. monocytogenes* 6550; *L. monocytogenes* 19114. Lanes 10-16: *L. monocytogenes* 15313; *L. welshimeri* 15897; *L. seeligeri* 35867; *L. ivanovii* 11846; *L. seeligeri* 1604; *L. innocua* 1909; negative control. Lanes 1 & 9 DNA molecular weight marker VI (size range: 0.15-2.1Kbp).

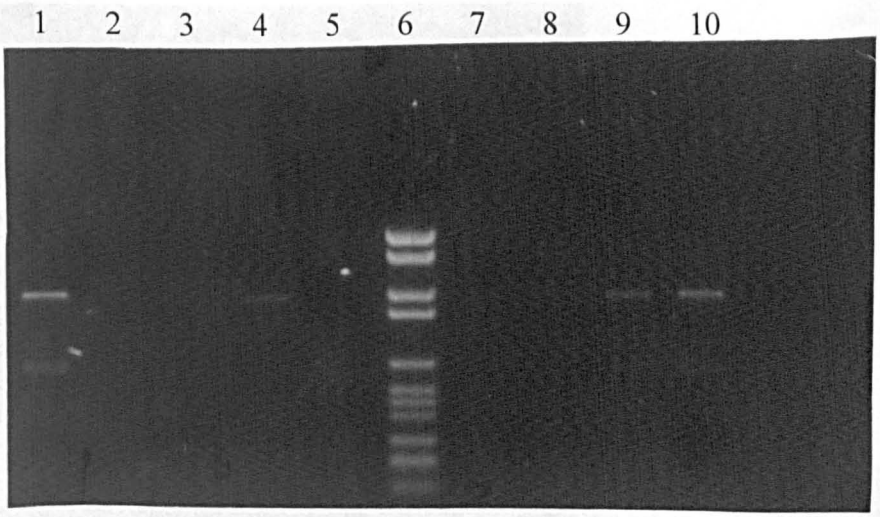


Figure 3.3.2.c. (upper). PCR amplification with primers MV2/MV9R annealing at 53 °C. Template DNA as follows (Left to right): *L. monocytogenes* 19114; *L. ivanovii* 11846; *L. grayi* 10815; *L. innocua* 33090; negative control; molecular weight marker VI (size range: 0.15-2.1Kbp.); *L. murrayi* 10812; *L. welshimeri* 15897; *L. seeligeri* 35967; *L. monocytogenes* 6550.

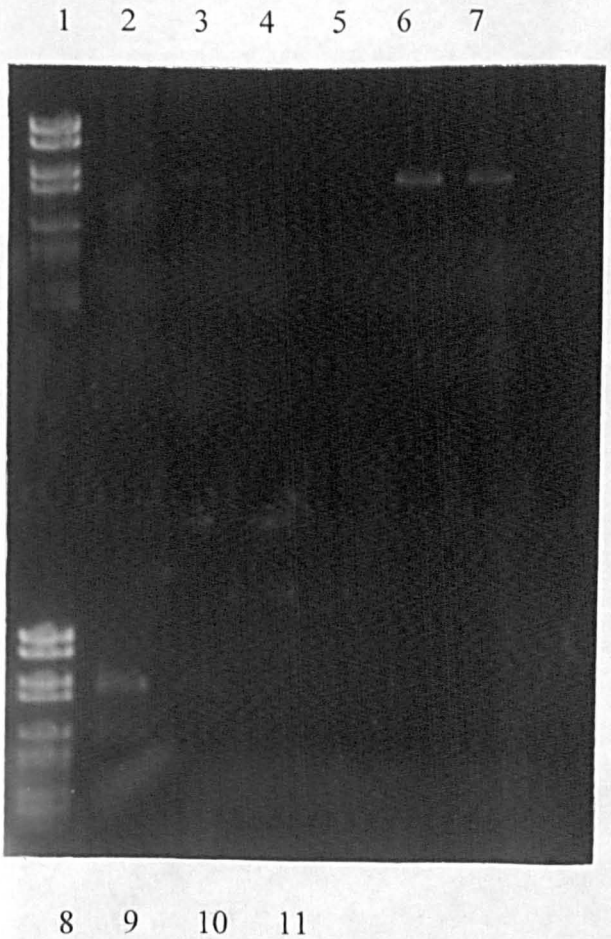


Figure 3.3.2.c: (lower). PCR amplification with primers MV2/MV9R annealing at 53 °C. Template DNA from left to right: *L. ivanovii* 11846; *L. monocytogenes* 19111; *L. grayi* 10815; *L. murrayi* 10812; *L. monocytogenes* 19113; *L. monocytogenes* 2073; *L. monocytogenes* 15313; *L. seelgeri* 15897; negative control. Lanes 1 & 8 molecular weight marker VI (size range: 0.15-2.1kbp.).

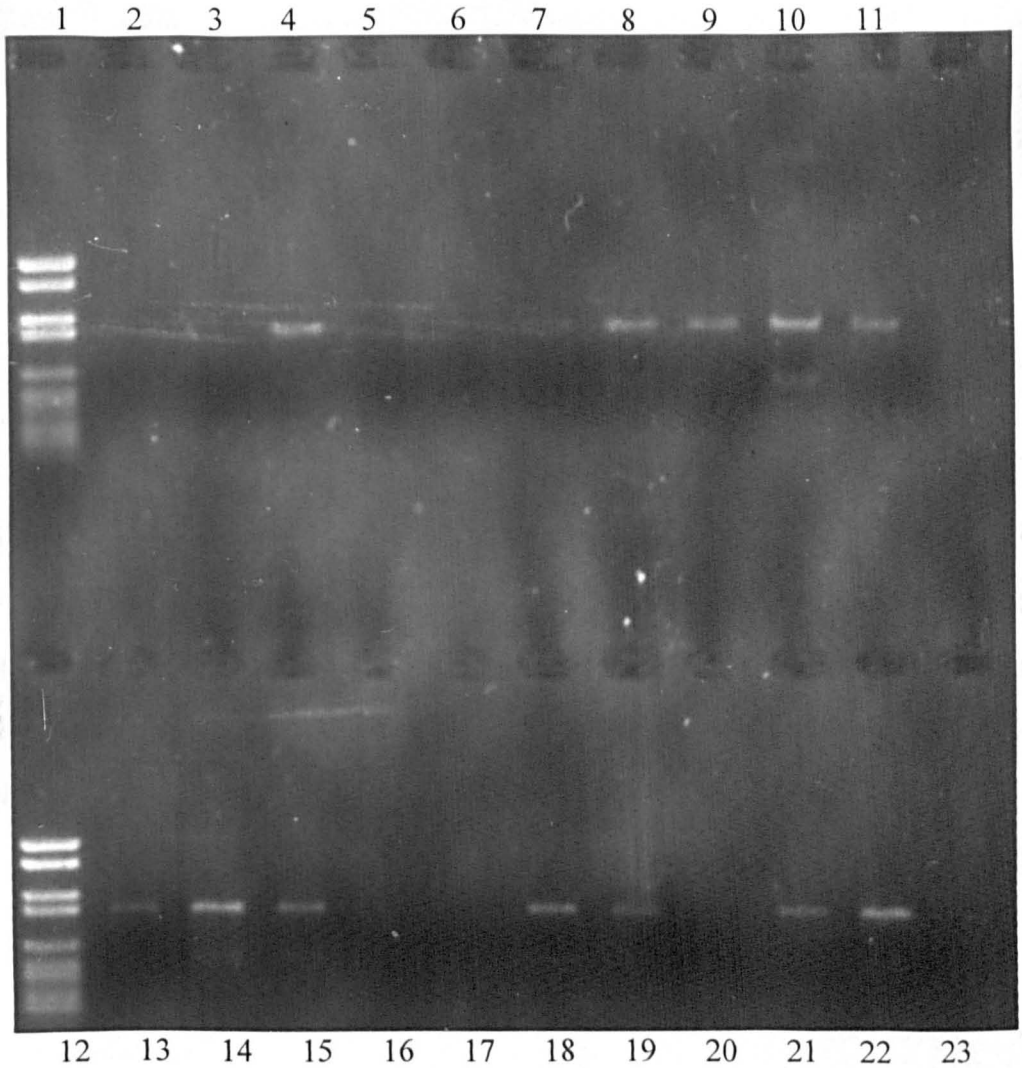


Figure 3.3.2.d. PCR amplification with primers MV2/MV9R annealing at 54°C. Template DNA from left to right: *L. seelgeri* 1604; *L. innocua* 2551; *L. monocytogenes* 1005; *L. innocua* 1909; *L. innocua* 2393; *L. innocua* 2393; *L. monocytogenes* 1800; *L. monocytogenes* 836; *L. monocytogenes* 6550; *L. monocytogenes* 2073; *L. monocytogenes* 19113; *L. innocua* 33090; *L. seeligeri* 35967; *L. grayi* 10815; *L. ivanovii* 11846; *L. monocytogenes* 15313; *L. monocytogenes* 19114; *L. monocytogenes* 19116, *L. monocytogenes* 9313; *L. monocytogenes* 19111, negative control. Molecular weight marker VI, (size range: 0.15-2.1Kbp) lanes 1 and 12.

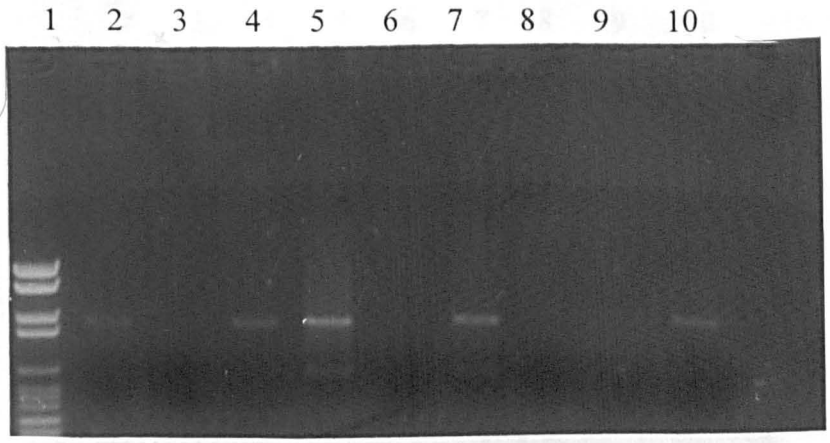


Figure 3.3.2.e. PCR amplification with primers MV2/MV9R annealing at 55°C. From left to right: Molecular weight marker VI (size range: 0.15-2.1Kbp.); *L. monocytogenes* 19114; *L. grayi* 10815; *L. monocytogenes* 19113; *L. monocytogenes* 19111; negative control; *L. monocytogenes* 15313; *L. monocytogenes* 2073; *L. seeligeri* 35867; *L. innocua* 33090.

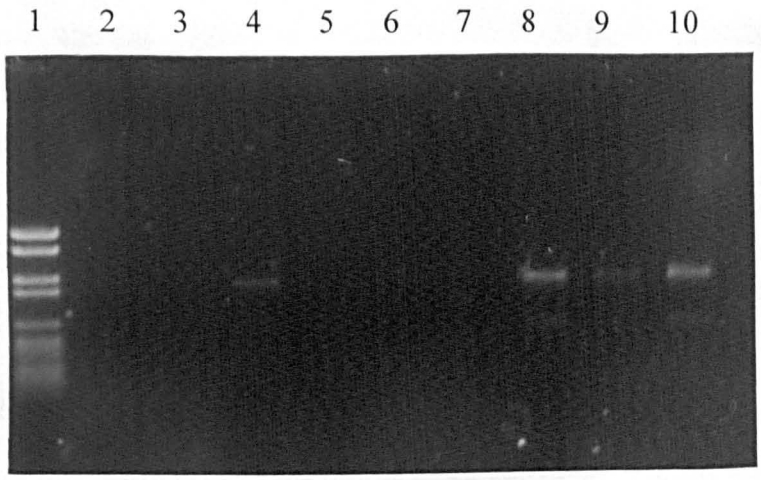


Figure 3.3.2.f. PCR amplification with primers MV2/MV9R annealing at 56°C. from left to right: DNA molecular weight marker VI (size range 0.15-2.1Kbp); *L. monocytogenes* 15313; *L. innocua* 1909; *L. inoccua* 33090; *L. seeligeri* 35967; negative control; *L. grayi* 10815; *L. monocytogenes* 19111; *L. monocytogenes* 19113; *L. monocytogenes* 9313.

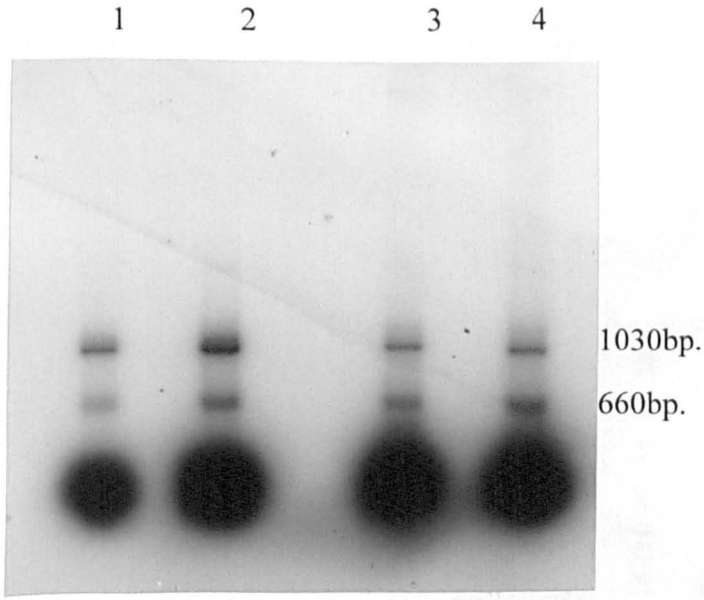


Figure 3.3.2.g. Incorporation of γ32-P-ATP labelled primers MV2 and MV9R into PCR products. Lanes 1 & 2 MV2; 3 & 4 MV9R.

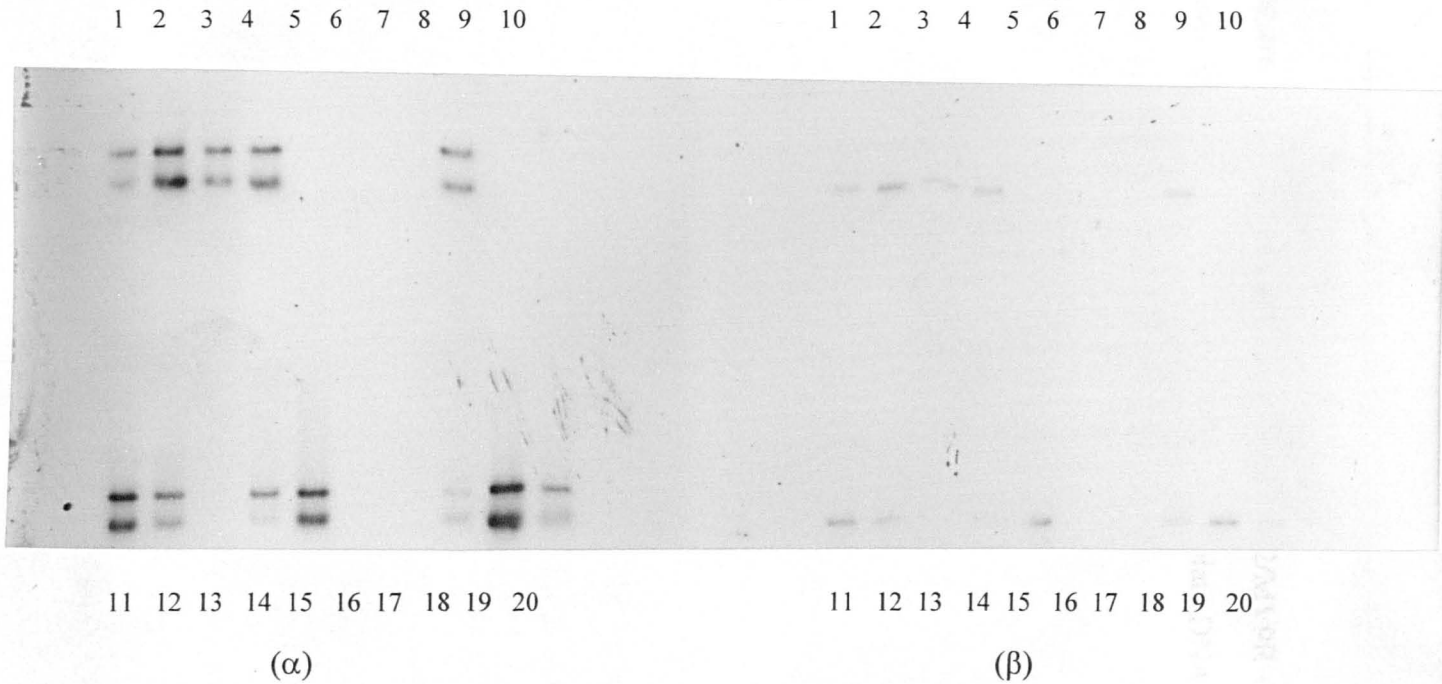


Figure 3.3.2.h. Confirmation of MV2/MV9R amplified DNA fragments source. DNA fragments applied to nylon membrane by alkaline transfer. Nucleic acid hybridisation performed overnight at 45°C with either 10pM radioactively labelled MV9RP2 (α) or MV9P (β). Template DNA (lanes 1- 10): 2073, 6550, 836, 1800, 2393, 7234, 1909, 1005, 2551, 1604; (lanes 11-21): negative control, 19111, 9313, 35967, 19114, 15313, 11846, 10815, 33090, 33090, 19113, negative control.

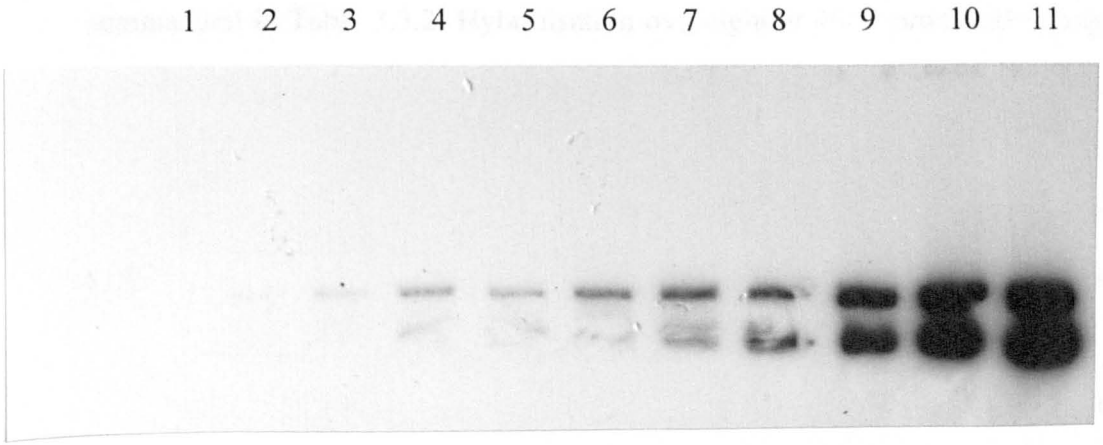


Figure 3.3.2.i. Sensitivity of primers MV2/MV9R demonstrated by titration of *L. monocytogenes* DNA. Picogrammes of template DNA from left to right. 0, 0.5, 1, 2 4 8, 16.3, 32.5, 65, 130, 265.

labelled oligonucleotide probe MV9RP2. The results from autoradiographs are summarised in Table 3.3.2. Hybridisation overnight at 40°C produced strong signals from solely *L. monocytogenes* derived target. At higher hybridisation temperatures, the intensity of positive results was seen to decrease. Hybridisation at higher temperatures reduced the relative intensity of *L. monocytogenes* DNA and templates derived from *L. innocua* 33090, 1909 and 2393 produced bands of an equal intensity relative to *L. monocytogenes* DNA.

When known concentrations of *L. monocytogenes* DNA were immobilised with a vacuum transfer manifold and hybridised overnight in the presence of either radio-labelled probes pA, MV9P or MV9RP2 at 40°C, the resulting autoradiograph was compared (Fig. 3.3.3). It is apparent that oligonucleotides pA and MV9RP2 have a greater sensitivity than MV9P. Consequently, subsequent quantitative DNA analysis employed either oligonucleotides pA or MV9RP2.

3.3.4. Development of quantitative oligonucleotide probing techniques.

Dilution series of known amounts of *E. coli* 16 and 23S rRNA were applied on to a nylon membrane with a vacuum transfer manifold. The filter was incubated overnight at room temperature in the presence of radio-labelled eubacterial specific oligonucleotide probe pE'. Autoradiographs (Fig. 3.3.4a) were exposed for 2 and 7 days. Scanning densitometry was used to assay the pixel intensity and the signal sum above background. Fig. 3.3.4b illustrates the relationship between nucleic acid concentration and sum above background. A significant signal was seen resulting from the lowest quantity of rRNA applied to the filter (11.5ng). This is too great to permit definition of the relationship between oligonucleotide probe binding and low concentrations of nucleic acid. Application of more than 10μg RNA results in a signal plateau where the filter has become saturated with RNA.

Table 3.3.2. Diagnostic probing of pA/pH' PCR products derived from *Listeria spp* DNA with oligonucleotide probe MV9RP2.

Strain No.	Species	Hybridisation temperature (°C)			
		40	45	55	60
15313	*L. monocytogenes*	+	+	+	+
19111	""	+	+	+	+
19113	""	+	+	+	+
19116	""	+	+	+	+
836	""	+	+	+	+
1005	""	+	+	+	+
1800	""	+	+	+	+
2073	""	+	+	+	+
6550	""	+	+	+	+
9313	""	+	+	+	+
33090	*L. innocua*	–	~	+	+
1909	""	–	~	+	+
2393	""	–	~	+	+
2551	""	–	–	–	–
7234	""	–	–	–	–
10815	*L. grayi*	–	–	–	–
11846	*L. ivanovii*	–	–	~	~
35967	*L. seeligeri*	–	–	–	–

N.B. + = Hybridisation band observed; – = negative; ~ = weak hybridisation observed.

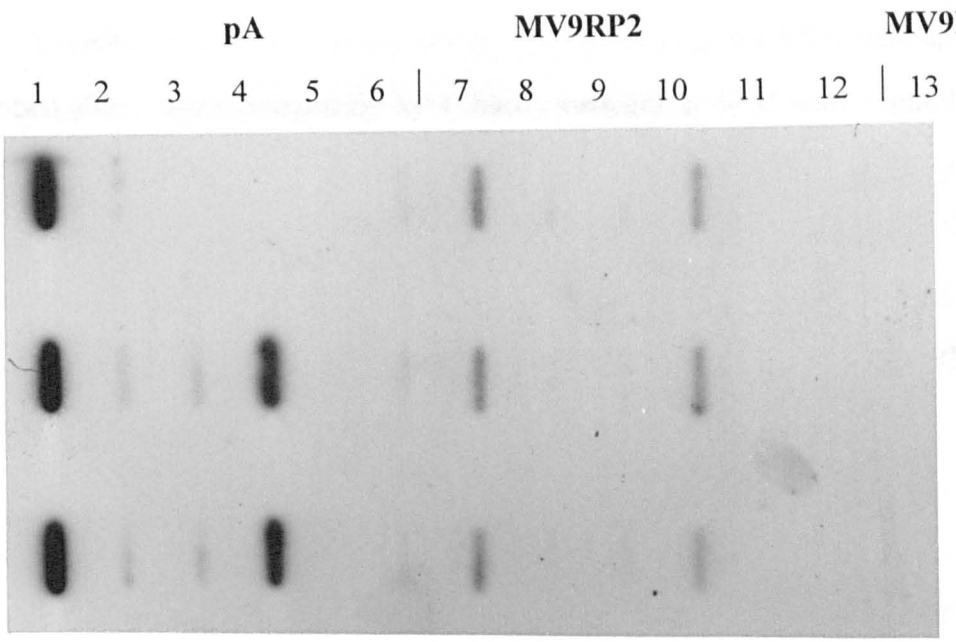


Figure 3.3.3. Assessment of sensitivity of oligonucleotide probes pA (columns 1-6), MV9RP2 (columns 10-12) and MV9P (column 13) on titration series of *L. monocytogenes* DNA. Hybridised overnight at 40°C. Left to Right: Columns 1- 3 and 7-9 & 13; 4.4, 0.44, 0.044 µl *L. monocytogenes* caesium chloride DNA preparation. Columns 4-6 and 10-12 & 13: 2.64, 0.264 and 0.0264µg proteinase K prepared *L. monocytogenes* DNA.

Titration series of known quantities of *L. monocytogenes* RNA were applied as described above and subsequently hybridised overnight at 40°C with 10pmol. radio-labelled oligonucleotide MV9RP2. The autoradiograph (Fig. 3.3.4c) exhibits a similar trend to that shown in Fig. 3.3.4a. Scanning densitometry analysis of this autoradiograph (Fig. 3.3.4d.) shows a similar relationship to that seen in Fig. 3.3.4b. However, it should be noted that the signal produced from less than 11.5ng RNA was below background levels.

L. monocytogenes DNA was applied to a nylon filter in an identical manner to that summarised above. This was hybridisied at room temperature with radio-labelled probe pA. The autoradiograph produced after incubation for 48h. at -70°C was developed and scanning densitometry performed to assess pixel intensity and sum above background. The autoradiograph and graph of sum above background against applied DNA are presented in Figs. 3.3.4e. and 3.3.4f., respectively. The relationship seen is similar to exhibited by RNA, but sensitivity is greatly reduced. Consequently, the sensitivity of DNA quantitation is significantly less in this example than that shown for RNA (1.32μg).

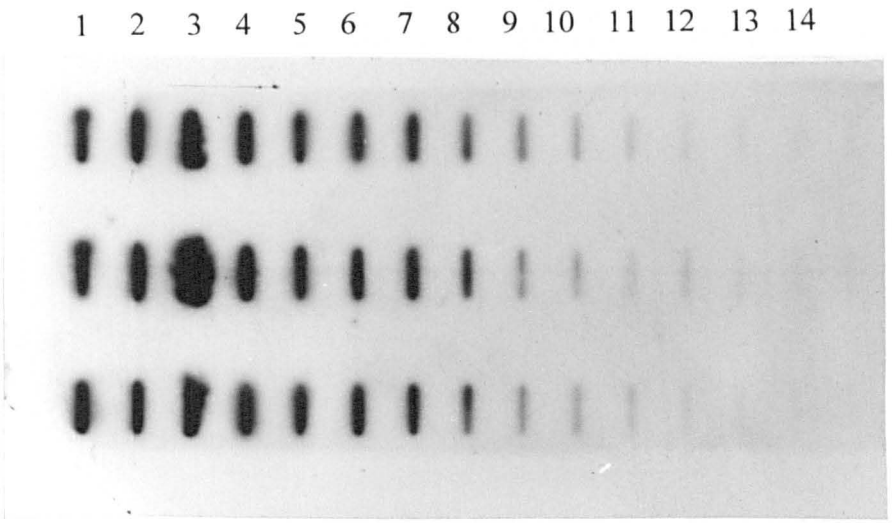


Figure 3.3.4.a. Titration of *E. coli* 16 and 23S rRNA hybridised overnight in the presence of 10pM radioactively labelled oligonucleotide probe pE'. Columns 1-14 (μg): 40, 20, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625, 0.0078, 0.0039, 0. N.B. Lane 3 (8μg) ommited from further analysis due to degradation of the slot manifold.

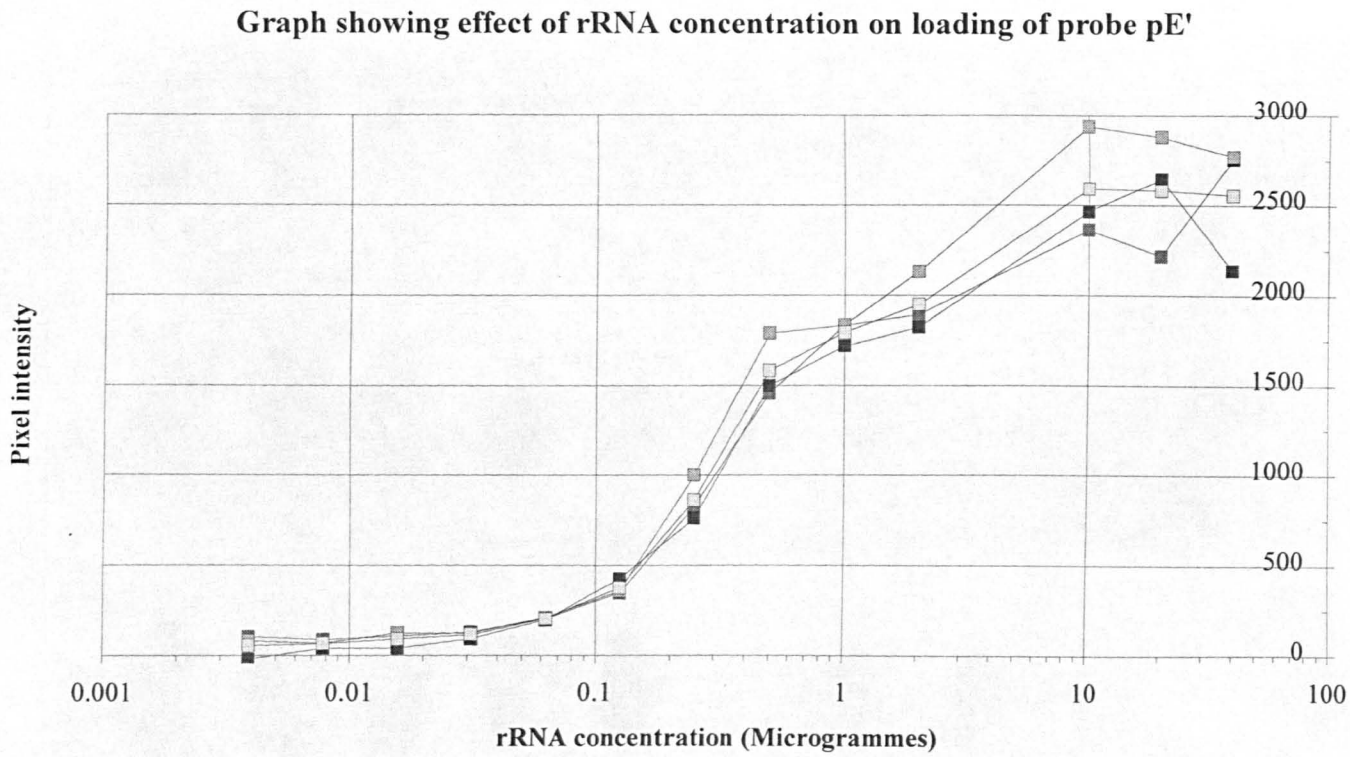


Figure 3.3.4b. Graph of Pixel intensity against *E. coli* 16 and 23S rRNA mixture concentration produced by scanning densitometry analysis of an autoradiograph after overnight hybridisation at 40°C with 10pmol. oligonucleotide probe pE'. RNA blotted in triplicate series and arithmetic mean are shown.

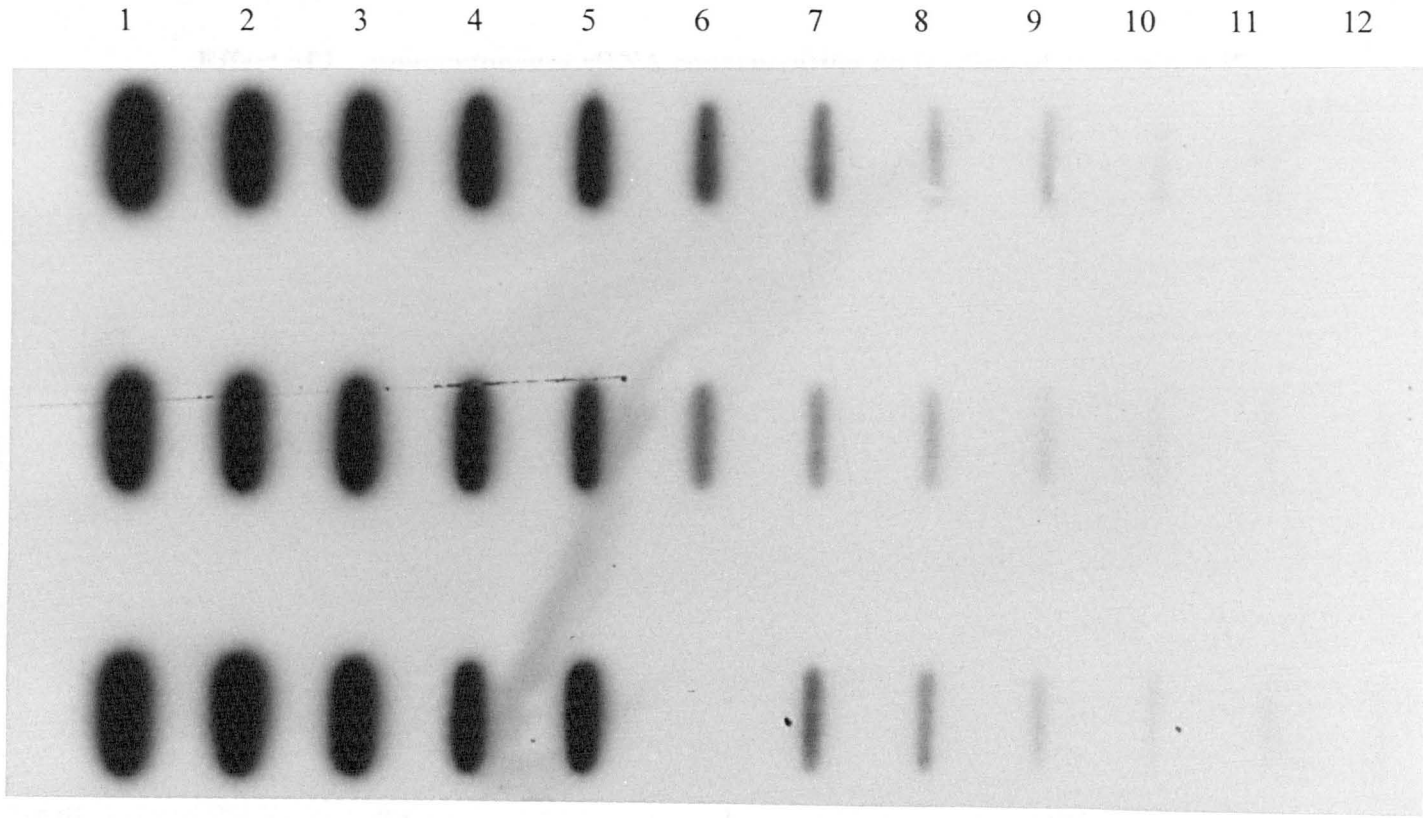


Figure 3.3.4.c. Titration of *L. monocytogenes* RNA hybridised at 40°C overnight in the presence of 10pM radio-labelled oligonucleotide probe MV9RP2. Concentration of RNA in ng from column 1-12: 3450, 2760, 2208, 1472, 736, 368, 184, 92, 46, 23, 11.5, 11.5.

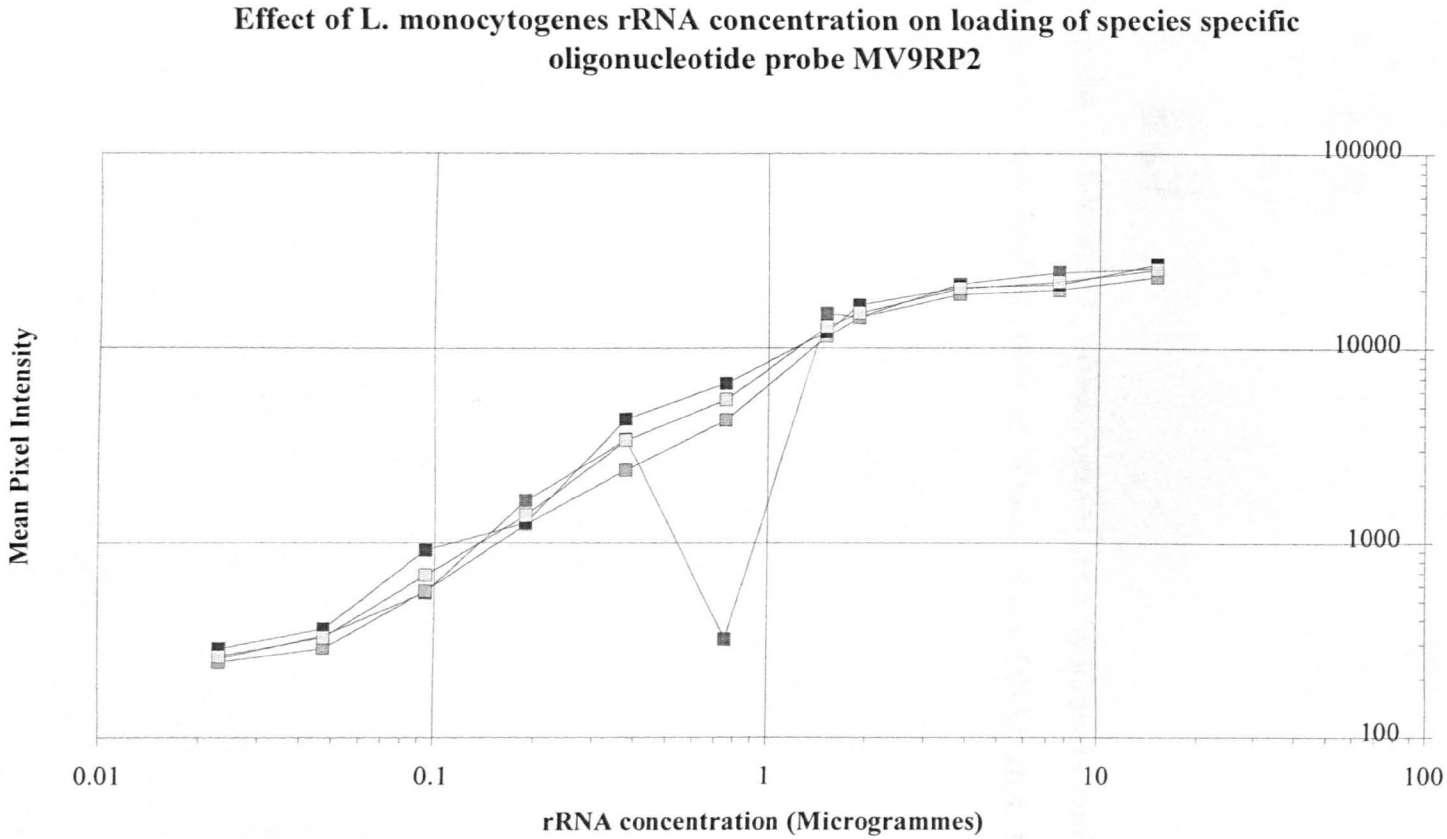


Figure 3.3.4d. Graph of pixel intensity against *L. monocytogenes* RNA concentration produced by scanning densitometry analysis of autoradiograph in Fig. 3.3.4c. RNA blotted in triplicate series and arithmetic mean shown.

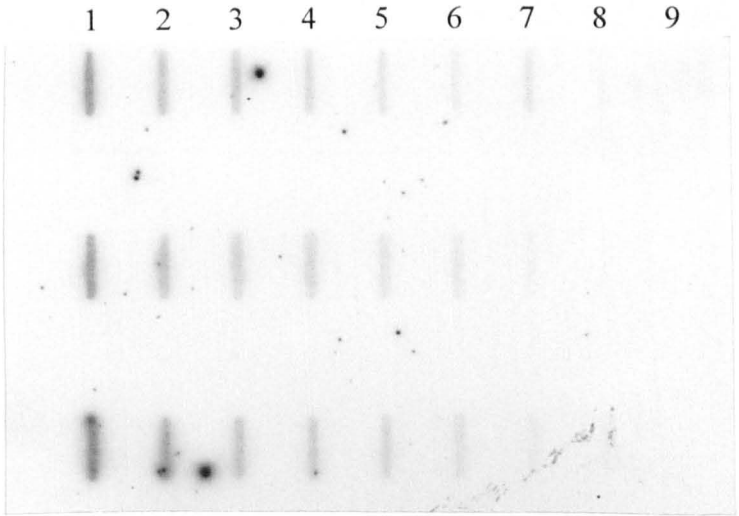


Figure 3.3.4.e. Titration of *L. monocytogenes* DNA hybridised overnight at 40°C with 10pM radioactively labelled probe pA. From 1-8 (μg DNA): 26.4, 19.5, 13.2, 10.56, 7.92, 5.28, 2.64, 1.32, 0.

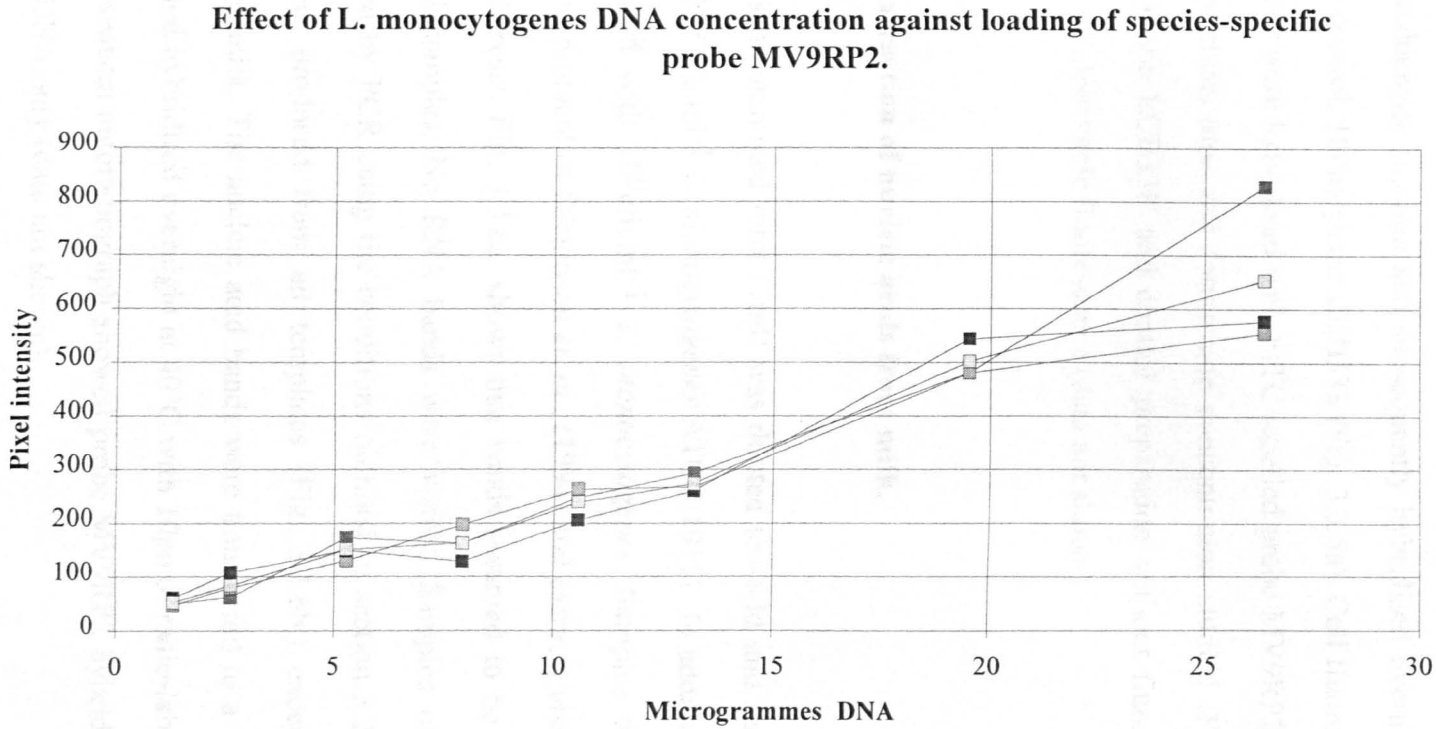


Figure 3.3.4f. Graph of Pixel intensity against *L. monocytogenes* DNA concentration as shown in autoradiograph, Fig. 3.3.4e. Traces represent replicate samples in triplicate series and arithmetic mean.

3.3.5. *In-situ* oligonucleotide probing of *L. monocytogenes* rRNA.

Mid-exponential phase cells of *L. monocytogenes* were prepared for *in-situ* hybridisation as described earlier (3.2.5.). Samples were examined by fluorescence microscopy and results recorded photographically. Fluorescence significantly above that produced from negative controls was observed only from samples fixed in paraformaldehyde solution and subsequently hybridised overnight at 37°C with the positive control, 100ng probe EUB338 (Fig. 3.3.5a). Cell fluorescence was negligible when cells were hybridised with FITC-labelled probe MV9RP2 under any conditions of temperature, time and formamide concentration studied (Fig. 3.3.5b.). Negative control probe EUB338' and control preparation without fluorescent probe showed little, or no observable fluorescence (data not shown).

3.3.6. Extraction of nucleic acids from milk.

Semi-skimmed milk (1ml) was diluted ten-fold and seeded with either 10^3, 10^6 or 10^9 cfu.ml^{-1} *L. monocytogenes* ATCC 19111. In addition 1ml undiluted milk was seeded with 10^9cfu.ml^{-1} *L. monocytogenes*. Samples were subjected to the extraction protocol of Dickinson *et. al.*, (1994) and extracts visualised by agarose gel electrophoresis. Fig. 3.3.6a. shows that bands assumed to be DNA were extracted from all samples. No RNA bands were visible. Samples of these extracts were amplified by PCR using the conditions outlined in section 3.2.3. Products of 1.5kb size were produced from all templates (Fig. 3.3.6b.) except that derived from undiluted milk. The nucleic acid bands were transferred to a nylon membrane (see 2.2.1.) and hybridised overnight at 40°C with 10pmol radio-labelled probe MV9RP2. The subsequent autoradiograph showed probe MV9RP2 hybridisation to the positive control DNA only (data not shown).

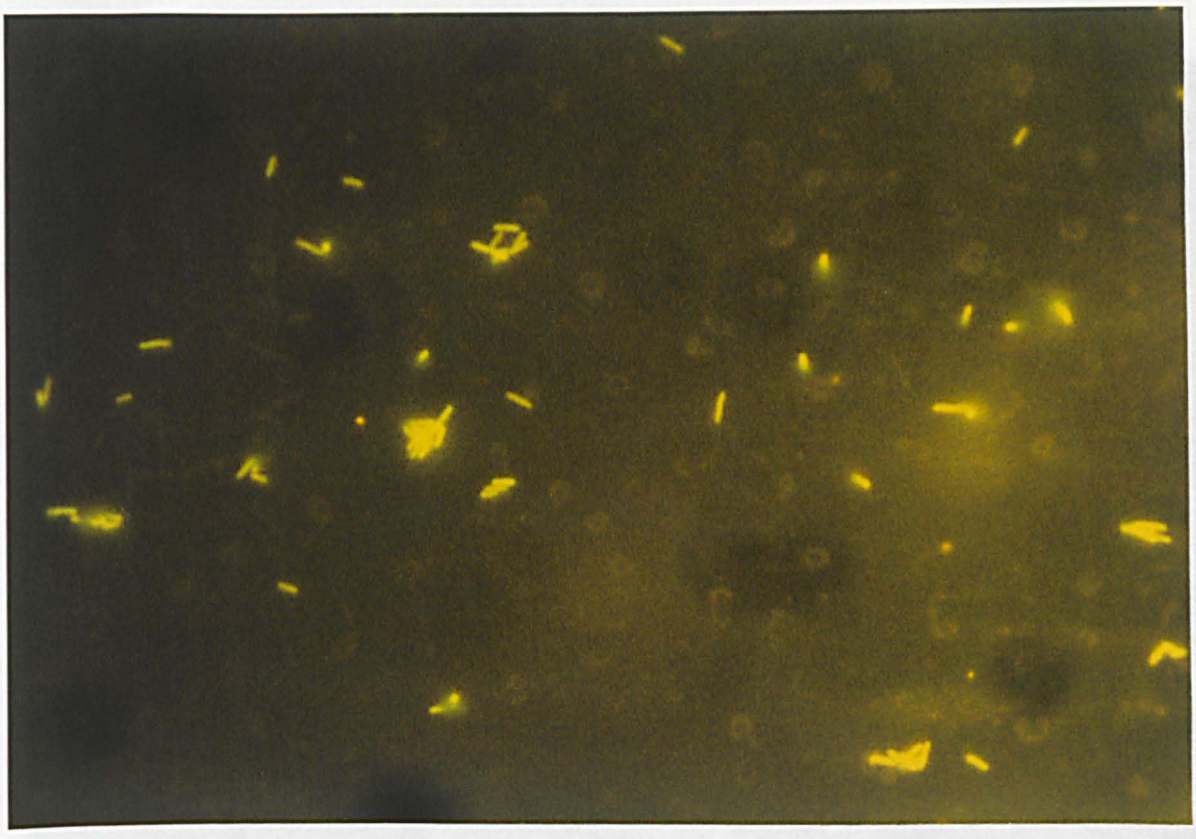


Figure 3.3.5.a. Photograph of *L. monocytogenes* cells hybridised for 16h at 37°C in the presence of FITC labelled oligonucleotide EUB338. Hybridisation buffer contained 40% (v/v) deionised formamide (X400).

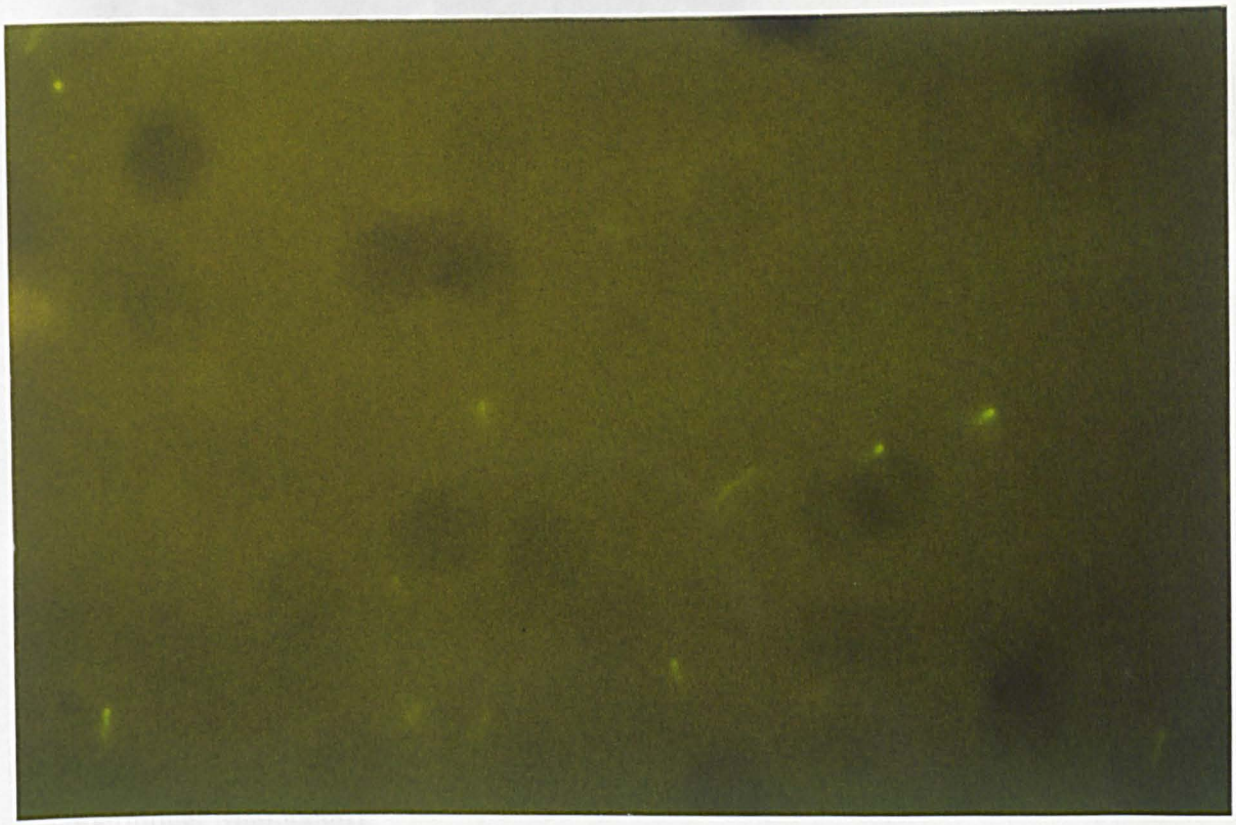


Figure 3.3.5.b. Photograph of *L. monocytogenes* cells hybridised for 16h at 37°C in the presence of FITC labelled MV9RP2. Hybridisation buffer contained 40% (v/v) deionised formamide (X400).

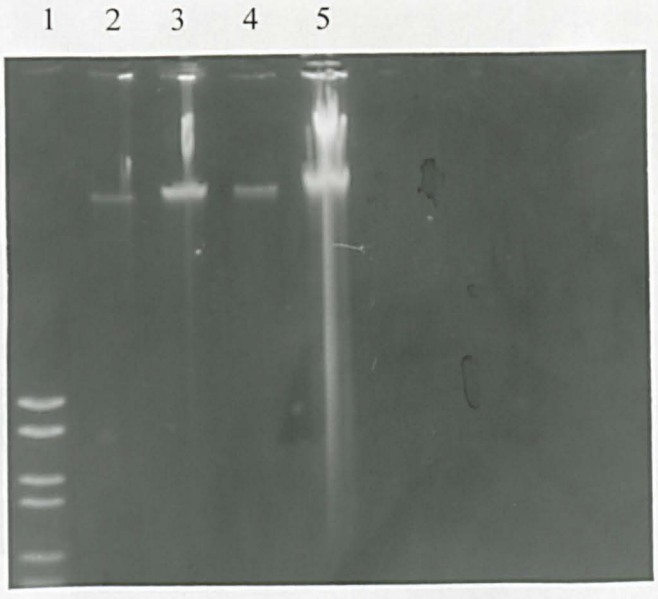


Figure 3.3.6a Nucleic acids obtained following the extraction procedure of Dickinson *et. al.*, (1993) from 10% (v/v.) milk seeded with either (Lanes 2-4) 10^3, 10^6 or 10^9 cfu. *L. monocytogenes* 19111. Lane 5: nucleic acids obtained by the same procedure as shown above from 1ml TSYGB broth culture. Lane 1: molecular weight marker VI (Boerhinger Mannheim).

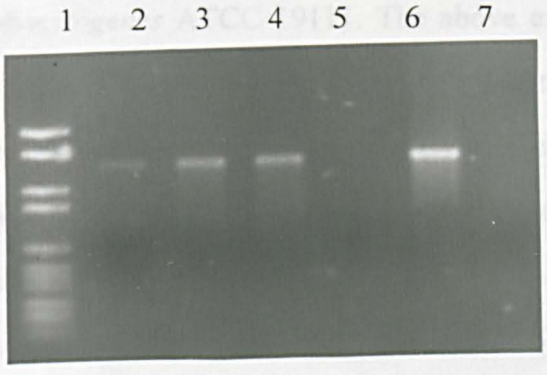


Figure 3.3.6b. Photograph of DNA fragments obtained from PCR amplification with eubacterial primers pA and pH' of nucleic acid templates shown above (Fig. 3.3.6a) Lane 1: DNA molecular weight marker VI; Lanes 2-4 DNA obtained from 10% (v/v) milk seeded with 10^3, 10^6 or 10^9 cfu. *L. monocytogenes.* Lane 5: DNA template obtained from neat milk seeded with 10^9 cfu. *L. monocytogenes.* Lanes 6 & 7 positive and negative controls, respectively.

Nucleic acid extraction protocols of Dickinson *et. al.*, (1994) and Catrimox™ were compared for obtaining both RNA and DNA from milk samples. Ten-fold dilution's of semi-skimmed milk were seeded with either 10^3, 10^6 or 10^9 cfu.ml^{-1} *L. monocytogenes* ATCC 19111. The above extraction procedures were performed on one ml aliquots of a 10% solution of milk and also one ml media (TSYGB) in which the cells had grown. Visualisation of the nucleic acid extracts (Fig. 3.3.6c) showed no nucleic acids derived from seeded milk. In contrast, the method of Dickinson *et. al.*, (1994) yielded bands tentatively identified as DNA and RNA extracted from the growth media. However, extraction by Catrimox™ from growth media did not result in recognisable nucleic acid bands when visualised by agarose gel electrophoresis.

3.3.7. Determination of the 16S rRNA gene copy number.

L. monocytogenes ATCC 19111 chromosomal DNA was cut with restriction endonucleases specific to sites outside the published 16S rRNA sequence and fragments separated. The autoradiograph produced after hybridisation with oligonucleotide probe pE' is shown in Fig. 3.3.7. Incubation in the presence of endonucleases *BamHI* or *ClaI* produced a maximum of 5 fragments ranging from approximately 12.3-3.9kb. in size. This suggests there are at least 5 copies of nucleotide sequence complementary to the oligonucleotide probes pE' present in *L. monocytogenes* 19111 chromosomal DNA, and consequently, 5 copies of the 16S rRNA gene.

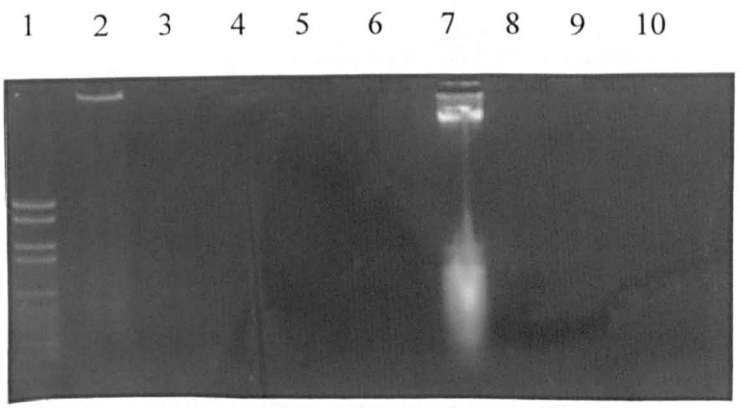


Figure 3.3.6c Comparison of techniques of Dickinson *et. al.*, (1993) and Catrimox™ (1994) for nucleic extraction from 10% (v/v.) solution of semi-skimmmed milk seeded with either 10^3, 10^6, or 10^9 cfu.ml^{-1} *L. monocytogenes* 19111. Lanes 3-5 (Catrimox™, 1994) and lanes 7-10 (Dickinson *et. al.*, 1993) Nucleic extraction was performed on growth media (TSYGB) containing 10^9 cfu.ml^{-1} *L. monocytogenes*, lanes 2 (Catrimox™, 1994) and 7 (Dickinson *et. al.*, 1993). Lane 1: DNA molecular weight marker VI.

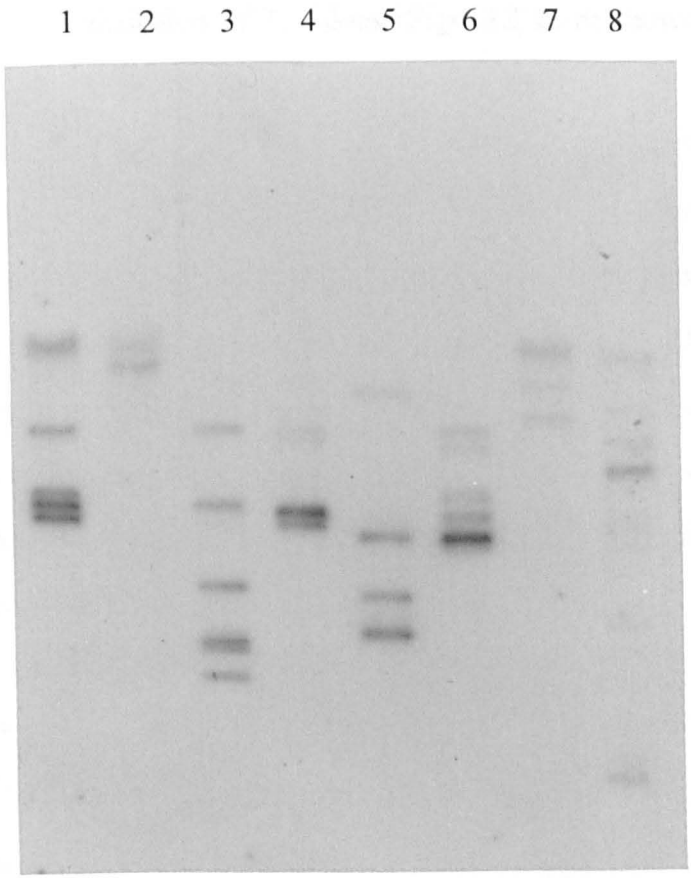


Figure 3.3.7. Determination of *L. monocytogenes* 16S rRNA gene copy number. *L. monocytogenes* ATCC 19111 DNA digested with the following restriction endonucleases; Left to Right: *BamH1, Stu 1, Cla I, EcoRV, Hae II, Hind III, Msc I* and *PvuII*. Fragments were immobilised on nylon membranes by standard procedures and hybridised overnight at 40°C in the presence of 10pM radiolabelled probe pE'.

3.4. DISCUSSION

3.4.1. Calculation of irreversible melting temperatures (T_i) for 16S rRNA oligonucleotide probes.

Calculation of T_i values (Figs. 3.3.1a-d) shows with the exception of SP, a sigmoidal relationship between temperature and percentage elution of the oligonucleotide under examination. A difference of 10°C between the theoretical Tm (53°C) and the experimental value of T_i (43°C) was observed for probe MV9RP2. Oligonucleotide probes MV9P and MV9RP2 were used to screen PCR fragments from 19 different *Listeria spp.*, with hybridisation at different temperatures. Results diagnostic for *L. monocytogenes* were obtained at 40°C for both probes studied. An increase in hybridisation temperature caused a decrease in positive signal intensity. The increased time required to develop autoradiographs from hybridisation temperatures above 40°C resulted in some hybridisation to *L. innocua* DNA derived from strains 33090, 1909 and 2393 (Table 3.3.2.). This cross reactivity was persistent at higher hybridisation temperatures studied (up to 60°C).

The discrepancy between theoretical and practical values of T_i for oligonucleotide MV9RP2 is probably due to non-specific probe binding which exhibits time dependent as opposed to temperature dependent characteristics (Hiorns, 1993). To clarify this discrepancy the experiment should be repeated at an increased starting temperature. The resulting graph should not be significantly displaced along the temperature axis. If displacement does occur this is probably due to a lower ratio of specific to non-specific probe binding. A plot of percentage probe elution against time would show dissociation to be time, rather than temperature dependent. The practical value T_i found for MV9RP2 was not diagnostic for *L. monocytogenes* exclusively, however an increase in hybridisation temperature (and therefore stringency) did not result in species-specific oligonucleotide probing. Published sequences of genus *Listeria* 16S rRNA (EMBL accession numbers 56148-56153;

Collins *et. al.*, 1989) indicate only two nucleotide differences between the two closest phylogenetic members, *L. monocytogenes* and *L. innocua* where MV9RP2 anneals (1281-1301bp.).

3.4.2. Evaluation of oligonucleotide PCR primers diagnostic for *L. monocytogenes.*

Theoretical values of T_m (Johnson, 1991) were 54° and 46°C for MV2 and MV9R respectively. Optimisation of PCR conditions with primer pair MV2 and MV9R by increases in annealing temperature showed persistent amplification of *L. innocua* ATCC 33090 over the range studied. Indeed, at a 56°C annealing temperature, *L. monocytogenes* 15313 was not amplified. Amplification was seen with less than 33pg template DNA in the reaction tube (Fig. 3.3.2.h). When a 'touchdown' PCR reaction (Don *et. al.*, 1992) was carried out *L. monocytogenes* type strain (ATCC 15313) only was amplified (data not shown), indicative of possible sequence divergence between strains of this species. The order in which *Listeria spp.* ceased to be amplified with primer pair MV2/MV9R when PCR annealing temperatures were sequentially raised and the phylogenetic relationship (in terms of 16S rRNA sequence) of genus *Listeria* members (Collins *et. al.*, 1991) show interesting similarities. At 45°C the annealing temperature of *L. grayi* and *L. murrayi* DNA do not amplify with this primer combination. These are the furthest species in phylogenetic terms (in terms of 16S rRNA sequence) from *L. monocytogenes.* Subsequent increases in annealing temperature do not amplify *L. welshimeri* and *L. ivanovii* (50°C); *L. seeligeri* (54°C) and most *L. innocua* strains tested (54°C). From the phylogenetic information of Collins *et. al.*, (1991) it would be predicted that the order of amplification "drop-out" with respect to increasing annealing temperature of this primer combination would be; *L. grayi* and *L. murrayi*; *L. ivanovii*; *L. seeligeri*; *L. welshimeri*; *L. innocua* and *L. monocytogenes* respectively. The experimental data

here supports the basic phylogenetic relationship between members of the genus *Listeria*, although further screening of isolates is required to test the relationship suggested by experimental data shown here.

L. monocytogenes-specific DNA amplification using 16S rRNA primers has been reported with varying degrees of success (Wang *et. al.* 1989; King *et. al.*, 1989). Indeed, it could be suggested that claims of species specificity are based on a fortunate selection of *Listeria spp* and serotypes for screening purposes. These difficulties are reported by Czjaka *et. al.*, (1993) who concludes the 16S rRNA V2 region (143-220bp.) to be of dubious diagnostic value for *L. monocytogenes* detection. These data indicate persistent cross-reactivity of *L. innocua* ATCC 33090 with PCR primers MV2 and MV9R at all temperatures examined. It is possible that further increases could have resulted in increased diagnostic amplification of *L. monocytogenes*, but further *L. monocytogenes* strains may not be amplified. It would be valuable (although tedious) to conduct exhaustive screening of 16S rRNA oligonucleotide probes and primers against *Listeria spp.* from both culture collections and environmental sources. Sequence divergence with respect to source and serotype of *L. monocytogenes* has been shown by both restriction fragment length polymorphism (RFLP) analysis (Harvey and Gilmour, 1994) and random amplified polymorphic dimorphisms (RAPD; Czajka, *et. al.*, 1993).

Primer pair MV2 and MV9R persistently amplified a double product band of the desired 1137bp. and also 660bp size. Manipulation of buffer pH, magnesium ion and Tris-HCl buffer concentrations did not alleviate this problem. In order to investigate further, both primers were end radio-labelled and suitable quantities incorporated into PCR reaction mixtures. Fig. 3.3.2.g shows that both forward and reverse primers were involved in this product formation. There are no other DNA fragments visible on this figure indicating the formation of primer-dimers unlikely. All amplifications with primers MV2 and MV9R employed 'hot-start' PCR (Chou *et. al.*, 1992), a procedure that has been shown to prevent pre-PCR mis priming and primer dimerisation.

Oligonucleotide probing overnight at 45°C of PCR fragments produced when DNA from 20 *Listeria spp.* was amplified with primers MV2 and MV9R, (annealing at 54°C) showed a similar pattern to that seen for MV9RP2 probing of pA/pH' PCR products. All strains of *L. monocytogenes* and *L. innocua* ATCC 33090 hybridised to both probes under the conditions studied. However, oligonucleotide probe MV9R appeared less sensitive than probe MV9RP2 despite approximately equal labelling efficiency. Curiously, probe MV9P only hybridised to the 660bp. product band from this reaction whilst MV9RP2 annealed to both fragments (Fig. 3.3.2.h). When either forward or reverse primers were used in combination with suitable 'universal' primers (pA or pE' for forward or reverse primer respectively) single product bands were detected (data not shown)

The origin of the 660bp. fragment was investigated by examination of 16S rRNA sequences (EMBL 56152-3) for regions outside the intended target where either primers could anneal and produce the desired product. No probable sites were found. Both large and small PCR fragments must contain the primer sequence MV9R and oligonucleotide probe MV9RP2 overlaps this sequence in 15 out of 21 nucleotides. These 15 nucleotides contain one nucleotide mis-match to other sequences of *Listeria spp.* (Collins *et. al.*, 1991). The remainder of this sequence (nucleotide positions 1281-1286) and the nucleotides immediately to the 5' side of the fragment sequence may be altered, or indeed missing to preclude probe MV9P binding. A minimum length of 15-17 nucleotides is recommended for oligonucleotide probes (Sambrook, *et. al.*. 1989). A sequence alteration at this site could prevent a DNA-DNA duplex forming due to either change or deletion of the nucleotide sequence. Species-specific primers may anneal to different copies of the 16S rDNA gene, one containing a mutation which is fortuitously amplified. This problem must be taken into consideration when DNA sequence information is obtained from the environment to ensure a representative sample of genotypes is extracted and sequence artefacts are not created by PCR (Amann, *et. al.*, 1995; Liesack, *et. al.*, 1991).

There have been no other reports of such a finding by other workers who have employed 16S rRNA sequences for the species-specific amplification and/or oligonucleotide probing of *L. monocytogenes*. Wang *et. al.*, (1991) successfully evaluated *L. monocytogenes* PCR primers based on the V9 region of 16S rRNA (1238-1304bp.). In this case, the reverse primer was from 1282-1301 and almost identical to that of MV9R. The origin of the secondary product band may be clarified if sequence data were obtained. The 16S rRNA sequence of the V9 region has been used successfully to detect *L. monocytogenes* exclusively by Ligase Chain Reaction (LCR) amplification (Wiedman *et. al.*, 1993). This technique exploits a single base pair difference to successfully amplify template DNA. Consequently, *L. monocytogenes* is an ideal target, containing few, and isolated differences in nucleotide sequence from *L. innocua*.

Reliable detection of only *L. monocytogenes* can be achieved by designing PCR primers which anneal to one of the genes implicated in pathogenicity. Oligonucleotides annealing to sectors of the haemolysin, (Blais *et. al.*, 1993; Bsat and Batt, 1993; Mengaud, *et. al.*, 1993 and Neiderhauser *et.al.*, 1992) delayed hypersnesitivity factor (Werners, 1991; Fluit *et. al.*, 1993) and *plcA* and *plcB* genes (Cooray *et. al.*, 1994) have been successful in this objective. However, detection of pathogenicity associated genes do not indicate the activity of the organsism.

3.4.3. Quantitative oligonucleotide probing techniques.

Graphs produced of pixel density above background from autoradiographs of either a RNA or DNA titration series, hybridised with oligonucleotide probes (Figs. 3.3.4a-f) show a similar relationship. At the lowest concentrations of nucleic acid applied, the pixel intensity is below background levels until a slow increase is observed. A linear portion follows where nucleic acid concentration is proportional to

the signal density until a plateau is reached. This plateau corresponds to saturation of the nylon membrane with nucleic acid (approximately 30μg per slot).

There are discernible differences between rRNA titration series (Figs. 3.3.4b and 3.3.4d). This is probably due to a combination of several factors. The pixel intensity shown in figure 3.3.4b rises from a background level of <100 units to approximately 12000 units, whilst that seen in Fig. 3.3.4d is from 200 to 3000units. Scanning densitometry of *L. monocytogenes* DNA dilution series hybridised with radio-labelled probe pA shows a proportional relationship between nucleic acid concentration and pixel intensity (Fig. 3.3.4f). It should be noted that the pixel intensities shown are significantly lower per unit DNA applied to the membrane than per unit RNA.

The differences in background levels may be due to non-specific probe binding or a consequence of increased lengths of time required for complete film development. Differences in pixel intensity at maximum nucleic acid loads may be due to variation in specific activity of radioactivity. Greater sensitivity at lower nucleic acid concentrations may reflect probe labelling efficiency or availability of target nucleic acids. A logarithmic relationship between pixel intensity and nucleic acid concentration is expected because of the nature of X-ray film reactivity. Radiation striking silver grains on photographic film initiates a chain reaction and so a doubling of probe binding should cause an exponential increase in film reaction (Sambrook *et. al.*, 1989). Apart from the general relationship between nucleic acid concentration and probe signal, comparison between autoradiographs is invalid due to the large number of variables involved when preparing filters and oligonucleotide probing. For example, differences may arise due to solution composition when immobilising nucleic acids, nucleic acid probing or autoradiograph development. In conclusion, quantitative oligonucleotide probing of immobilised environmental nucleic acid samples may be achieved by simultaneous application of standard nucleic acids (of known concentration) to a membrane before probe hybridisation reactions and autoradiography.

The quantity of target nucleic acid sequence applied to each filter will determine the limit of detection of a given nucleic acid probe. As the length of 16 and 23S rRNA of both *E. coli* and *L. monocytogenes*, and *L. monocytogenes* genomic DNA can be estimated it is possible to calculate the percentage potential target for an oligonucleotide probe. The *E. coli* 16 and 23S rRNA contain approximately 0.4% of target RNA whereas the *L. monocytogenes* DNA extract consists of 0.004% target. The *L. monocytogenes* RNA extracts immobilised on nylon filters and hybridised with MV9RP2 or pE' contain approximately 0.44% target. This explains, in part why the detection limit of slot-blot immobilised RNA and DNA are ≤60ng and ≤1μg respectivley.

3.4.4. Optimisation of *in-situ* rRNA oligonucleotide hybridisation.

Hybridisation of *L. monocytogenes* cells was performed in the presence of either oligonucleotide probe EUB338 or MV9RP2 under varying formamide concentrations and lengths of time. Fluorescence was observed when cells were stained with EUB338 only (Fig. 3.3.5 a-b) for 12 h. in the presence of 40%(v/v) formamide. Alteration of the formamide concentration and hybridisation time had little, if any effect on cell fluorescence due to hybridisation of oligonucleotide probe MV9RP2 to the cellular rRNA.

Low fluoresence from *in-situ* hybridisation with fluorescently labelled oligonucleotides is largely attributable to either poor cell permeabilsation, inaccessibility of the target site, hybridisation at a temperature above the Tm value, poor probe labelling; or a combination of the above factors (Amman, 1995).

Results described here suggest that *L. monocytogenes* cells were sufficiently permeabilised to allow hybridisation of probe EUB338. Permeabilisation in 37%(v/v) filtered formaldehyde and PBS buffer has been used extensively for Gram-negative organisms (DeLong, 1989), but reported to be detrimental in Gram-positive cell

fixation prior to *in-situ* hybridisation (Braun-Howland *et. al.*, 1992). Fixation of cells overnight in 50% (v/v) ethanol solution was attempted in the course of these studies as recommended for Gram-positive organisms (Roller *et. al.*, 1994) but little visible difference was observed when compared to formaldehyde fixed cells.

Little information is available regarding target site accessibility of probe MV9RP2, indeed it is suggested that minimal change in probe target site can cause alterations in probe annealing efficiency (Amann, 1995). However, the use of solvents such as formamide in hybridisation solutions aids relaxation of rRNA secondary structure (Amann, 1995), RNA/RNA interactions and RNA/protein interactions by weakening hydrogen bonds.

Oligonucleotide MV9RP2 has been employed as a radioactively labelled probe on rRNA and DNA extracts at temperatures ranging from 40 to 60°C with success. Consequently, the *in-situ* hybridisation temperature used here was not outside this range. The FITC-labelled oligonucleotide probe used was obtained commercially and included both labelling efficiency and concentration statistics, which were found to be satisfactory.

3.4.5. Nucleic acid extraction from milk.

The two nucleic acid extraction procedures evaluated to obtain both DNA and RNA from milk were of limited success. A cationic surfactant solution, (Catrimox™) produced distinct bands of DNA when *L. monocytogenes* cells in growth media were lysed. No RNA bands were seen from Catrimox™ prepared nucleic acid extracts from milk. This reagent has been reported to extract mRNA suitable for reverse transcriptase PCR (RT-PCR) from both blood and serum samples (Macfarlane and Dahle, 1993). Extraction of DNA from milk by the method of Dickinson *et. al.*, (1994) was successful at all levels of *L. monocytogenes* added (Fig. 3.3.6.a.). No RNA bands were seen. PCR amplification of this DNA with eubacterial primers was

achieved (Fig. 3.3.6.b). However, transfer of these fragments on to a nylon membrane and oligonucleotide probing with labelled MV9RP2 suggested that these DNA fragments were not *L. monocytogenes* in origin by absence of positive hybridisation.

A principal objective of this investigation was the extraction of both RNA and DNA from bacterial cells added to milk. If this objective had been attained it would have been possible to quantify these molecules, and gain information relating to the physiological state of the organism *in-situ*. Adaptation of the Catrimox™ to extract both DNA and RNA was investigated by precipitation with Phenol:Chloroform:Isoamylalcohol at pH 8.0, and not acidic pH values as recommended (Catrimox™, 1993). The method of Dickinson *et. al.*, (1994) involved lengthy incubation steps which could lead to RNase degradation although this phenomena was investigated by analysing nucleic acid extracts at each stage of the published extraction protocol with little, or no degradation observed (data not shown).

L. monocytogenes nucleic acids have been successfully extracted from food samples by a variety of methods. Cell separation from the matrix by immunomagnetic beads (Skerjve *et. al.*, 1990), washing and differential centrifugation (Cooray *et. al.*, 1994) or agglutination on lectin-coated Dynal™ beads (Grant et. al., 1993) have been used to separate cells from food material prior to nucleic acid extraction. Alternatively, nucleic acids from food samples may be directly precipitated with Polyethyleneglycol (PEG) and Dextran bead two-phase separation techniques (Lantz *et. al.*, 1994) or sodium iodide precipitation (Makino *et. al.*, 1995). Low detection limits, lack of universal methods for cell separation from commonly examined foodstuffs, have been barriers to employment of these techniques. This problem is accentuated in the presence of competing microorganisms (Grant, *et. al.*, 1993) which are found in many foodstuffs. Separation and collection of sufficient cells from foodstuffs to allow assessment of RNA and DNA levels would provide valuable *in-situ* measurements of cell activity.

3.4.6. Determination of 16S rDNA gene copy number.

The results presented in Fig. 3.3.7. suggest that there are five copies of the 16S rRNA gene. This is identical with replicate DNA extracts from the same *L. monocytogenes* strain, and indistinguishable banding patterns were observed when fragments were probed with either pE' or MV9RP2. Comparison of copy number determined here with that of other organisms shows this to be less than *E. coli* (7; Jinks-Robertson and Nomura, 1987), *Lactococcus lactis* (6; Beresford and Condon, 1991) and *Bacillus subtilis* (10; LeFauci, *et. al.*, 1986). However, the copy number is greater than slow growing organisms, for example, *Mycoplasma* and Ammonia oxidising bacteria (2; Ji, *et. al.*, 1994 and Hiorns, personal communication) and *Pseudomonas stutzeri*, (4; Kerkhof and Ward, 1993). The copy number of the 16S rRNA gene been determined previously to be 6 (Michel and Cossart 1992) and 5 (Carriere *et. al.*, 1991). Given the rapid growth rates shown by *L. monocytogenes* the figure obtained in this study supports the hypothesis that microorganisms capable of rapid growth posess increased rRNA gene numbers with respect to slow growing ones (Amikam *et. al.*, 1982; Nomura *et. al.*, 1977). It is impossible to assess how many gene copies are transcribed at any given instance, if at all, from the data shown here. Some copies of the gene may be preferentially transcribed over others depending on environmental conditions. Indeed, the gene copy number may be of less significance than their proximity to the chromosomal origin of replication. In *E. coli* there are 7 16S rRNA gene copies, but in rapidly dividing cells (2.5 doublings per h.) there may be between 12 and 36 copies of the rRNA genes due to multiple chromosome forks (Bremer and Dennis, 1987). However, this does not explain the discrepancy between rRNA genes and rRNA synthesis rates highlighted by Jinks-Robertson (1987) Individual rRNA operons may have divergent control region sequences, or control mechanisms resulting in different rates of gene expression for each operon (Wagner, 1994).

The method demonstrated here has been successfully used to determine the 16S rRNA gene copy number for *E. coli* (Kiss, *et. al.*, 1977; Michel and Cossart 1992). Confirmation of this would require further enzyme digests with other restriction endonucleases shown to cut DNA outside the 16S rRNA sequence. Considerable care needs to be exercised in the separation of DNA fragments by agarose gel electrophoresis as very small differences in fragment size have to be detected.

Further studies on 16S rRNA gene copy number could be done on different *Listeria spp.* and *L. monocytogenes* serotypes from culture collections and patient isolates. This would examine differences in gene copy number between species and serotypes and investigate any sequence divergence which may arise. This may lead to banding patterns of diagnostic value.

CHAPTER 4.

OPTIMISATION OF NUCLEIC ACID EXTRACTION TECHNIQUES FOR THE DETECTION OF *Listeria monocytogenes*.

4.1. Aims.

- To establish the efficiency and reproducibility of various cell lysis procedures for extraction of nucleic acids from *L. monocytogenes.*

- To develop an extraction procedure that would yield DNA and rRNA simultaneously, preferably as a single tube process to permit quantitative data on RNA/DNA ratios to be obtained.

4.2. Materials and Methods.

4.2.1. Strains and Culture Conditions. Cultures of *L. monocytogenes* ATCC 19111 were grown for 16 h. (late exponential to stationary phase; ca. 10^9 cells.ml^{-1}) at 37°C in TSYGB as described in chapter 2. Cells were harvested by centrifugation at 275g for 15 min, washed twice and resuspended in 1 ml phosphate buffered saline (PBS) at pH 7.4 (8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4 dissolved in 800ml distilled H_2O and adjusted to pH 7.4 before making up the volume to one litre with distilled water). In all experiments, a comparison was made between cells that had been fixed for one hour in 1 ml 70% (v/v) ethanol at 4°C prior to washing and resuspension in the appropriate buffer, and cells which had not been fixed in ethanol.

4.2.2. Fluorometric assay for nucleic acids and preparation of cell suspensions.

Staining solution (10 mg.ml^{-1} ethidium bromide in 5 mM Tris. HCl pH 8.0, 10 mM EDTA) was added to cell suspensions or cell extracts whose volume had been made up to 3 ml with PBS, to give a final concentration of 10 μg.ml^{-1} ethidium bromide. The

reaction mixtures were incubated for 15 min at 37°C and fluorescence measured with a Perkin Elmer 3000 Spectrofluorometer (excitation λ 260 nm, emission λ 590 nm; LePecq and Paoletti, 1966) using slit widths of 10 nm and 20 nm for excitation and emission respectively. All solutions were prepared in Hypersolv water (BDH). Expansion of signals was not required throughout, and all solutions were checked for quenching or enhancement of fluorometric readings. Unstained cells were resuspended in PBS without ethidium bromide as an additional control. In all controls, fluorescence was negligible in the absence of nucleic acid.

Standard curves for the fluorometric determination of DNA and RNA were prepared using serial dilutions of chromosomal DNA from *L. monocytogenes* ATCC 19111 (undiluted concentration 5.68 $\mu g.\mu l^{-1}$ determined by A_{260}) and 16S and 23S rRNA ($4\mu g.\mu l^{-1}$, Boerhinger Mannheim 206938) in Hypersolv water. The data were subjected to linear regression analysis.

4.2.3. Release of nucleic acids by physical disruption of *L. monocytogenes.*

Cell pellets were resuspended in 1 ml of a buffer that contained 100µl of 10% (v/v) Tris EDTA buffer (10mM Tris-HCl, 1mM EDTA, pH 8.3), 100µl of 10% (v/v) Tween 80 and 800 µl Hypersolv water. These preparations were incubated in a water bath at 100°C for 10 min, and then rapidly frozen in liquid nitrogen. This cycle was repeated up to three times and the effect of the number of freeze-thaw cycles on nucleic acid release determined. The freeze-thawed suspensions were centrifuged at 9440g for 15 min, the supernatant fluids carefully removed and both pellets and supernatant fluids suspended in staining mixture for fluorometric analysis as described above.

Cell pellets were also disrupted by ultrasonication. Samples (1ml) of *L. monocytogenes* cells in PBS were sonicated on ice in an ultrasonicator (MSE, Loughborough, England) for up to five cycles of treatment. Each cycle comprised 30 seconds at 18 µm peak to peak, followed by a pause of 30 seconds. The ultrasonicated

suspensions were centrifuged at 9440g for 15 min and the supernatant fluids and pellets separately stained at 37°C for 15 min for fluorometric analysis. The effect of the number of ultrasonication cycles and the duration of each cycle on nucleic acid release was determined.

Mean fluorescence values for each treatment were compared using a two-tailed significance test. A sample result leading to the rejection of the null hypothesis (H_0; that all means were the same) at 5% significance level was used throughout.

4.2.4. Release of nucleic acids by enzymatic lysis of *L. monocytogenes.*

1) Mutanolysin. Pellets of ethanol-fixed and unfixed cells were resuspended in 200µl mutanolysin buffer (50mM D-glucose, 10 mM EDTA, 25 mM Tris.HCl pH 7.3) (Siegal *et. al.* 1981), and 100 µl 0.5M EDTA and 20 µl of mutanolysin solution (mutanolysin, Sigma M9901 resuspended in mutanolysin buffer to 200 $U.ml^{-1}$) added to each. These were incubated at 37°C for different periods of time and 60 µl of 50 mM sodium deoxycholate added to lyse the cells prior to staining for fluorometry as described above.

2) Achromopeptidase. A similar procedure to that described for mutanolysin treatment was followed except that the cell pellets were resuspended in 200 µl achromopeptidase buffer (0.01M NaCl, 0.01M Tris.HCl pH 5.3), and 20 µl of a 10 U μl^{-1} solution of achromopeptidase (Sigma A3422) added.

3) Proteinase K. Cell pellets were resuspended in 200 µl of proteinase K buffer (50 mM Tris.HCl pH 8.0, 1 mM $CaCl_2$ 1% (w/v) sodium dodecylsulphate) and Proteinase K, (Boehringer Mannheim 161 519) added to a final concentration of 500 $\mu g.ml^{-1}$. These were incubated at 55°C for various durations and treated as described for mutanolysin .

4) Lysozyme. Pellets of ethanol-fixed and unfixed cells were resuspended in 200 µl of ice-cold lysozyme buffer (50 mM D-glucose, 10 mM EDTA, 25 mM Tris.HCl pH8.0) and 100 µl of 1mM EDTA pH 8.2. Lysozyme (Sigma L6876) was dissolved in lysozyme buffer to a concentration of 10 $mg.ml^{-1}$ and 100 µl added before incubation at 37°C for different periods of time. Samples were then analysed as described for mutanolysin.

4.2.5. The effect of RNase and DNase-treatment on fluorescence of whole cell suspensions and cell extracts.

Whole cell pellets resuspended in staining solution as described above, were treated with RNase and fluorescence monitored at hourly intervals for 5h. DNase-free RNase (Boehringer Mannheim 1119915) was added (3-15 $U.\mu l^{-1}$) and preparations incubated at 37°C. Cell pellets were also resuspended in 3 ml DNase buffer (20 mM Tris.HCl and 10 mM $MgCl_2$ pH 7.4) and fluorescence monitored at hourly intervals for 5 h. after the addition of 3 µl RNase-free DNase (Boehringer Mannheim 776785).

Listeria monocytogenes cell suspensions disrupted by three cycles of freeze-thaw treatment were also examined for depletion of fluorescence during incubation with RNase. Triplicate samples containing 1.0 x $10^9 cfu.ml^{-1}$ were used and fluorescence determinations performed on both supernatant fluids and pellets.

4.2.6. Extraction and electrophoresis of nucleic acids.

Cell suspensions lysed by physical or enzymatic treatments were treated as follows. Dowex silicone grease (60µl) was added (Mukhopadhyay and Roth 1993) followed by addition of 1 ml phenol:chloroform:isoamylalcohol (25:24:1). The

preparations in 1.5 ml eppendorf tubes were mixed by inversion and then centrifuged at 9440g for 5 min at room temperature. The aqueous phase was removed, 1 ml absolute ethanol added and the preparations held on ice for 1 h. Nucleic acids were pelleted by centrifugation at 9440g for 30 min at 4°C. The pellet was resuspended in 200 µl TE buffer pH 7.4 (10 mM Tris.HCl pH8.0, 1 mM EDTA) and 12 µl aliquots loaded on to 1 % (w/v) agarose gels for electrophoresis at 90 $V.cm^{-1}$.as described in chapter 2.

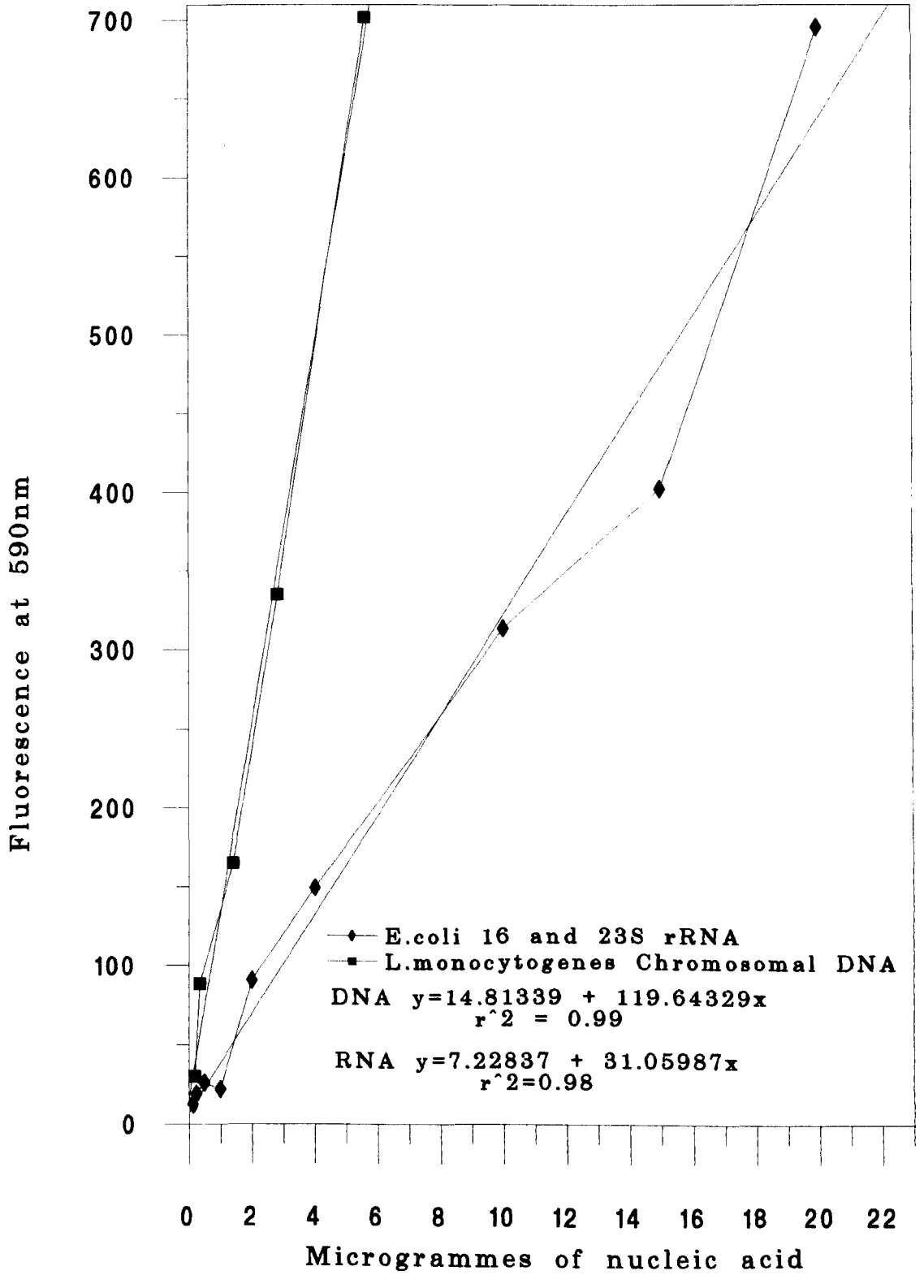
4.3. Results.

4.3.1. The relationship between fluorescence and nucleic acid concentration.

Both pure preparations of *L monocytogenes* DNA and the *E. coli* rRNA preparation (Boerhinger Mannheim 206938) gave a linear relationship when nucleic acid concentration was plotted against fluorescence at 590 nm in the presence of ethidium bromide (Fig. 4.3.1.). Linear regression analysis on the standard curve for rRNA gave values for the intercept of 7.2, the gradient 31.06 and an r^2 value of 0.976. Regression analysis of the DNA standard curve gave values of 14.81, 119.6 and 0.993 for intercept, gradient and r^2 respectively. The ratio of the slopes DNA/rRNA was 3.9. For a given concentration of nucleic acid, DNA would therefore be 3.9 times more fluorescent than rRNA on the basis of these preparations.

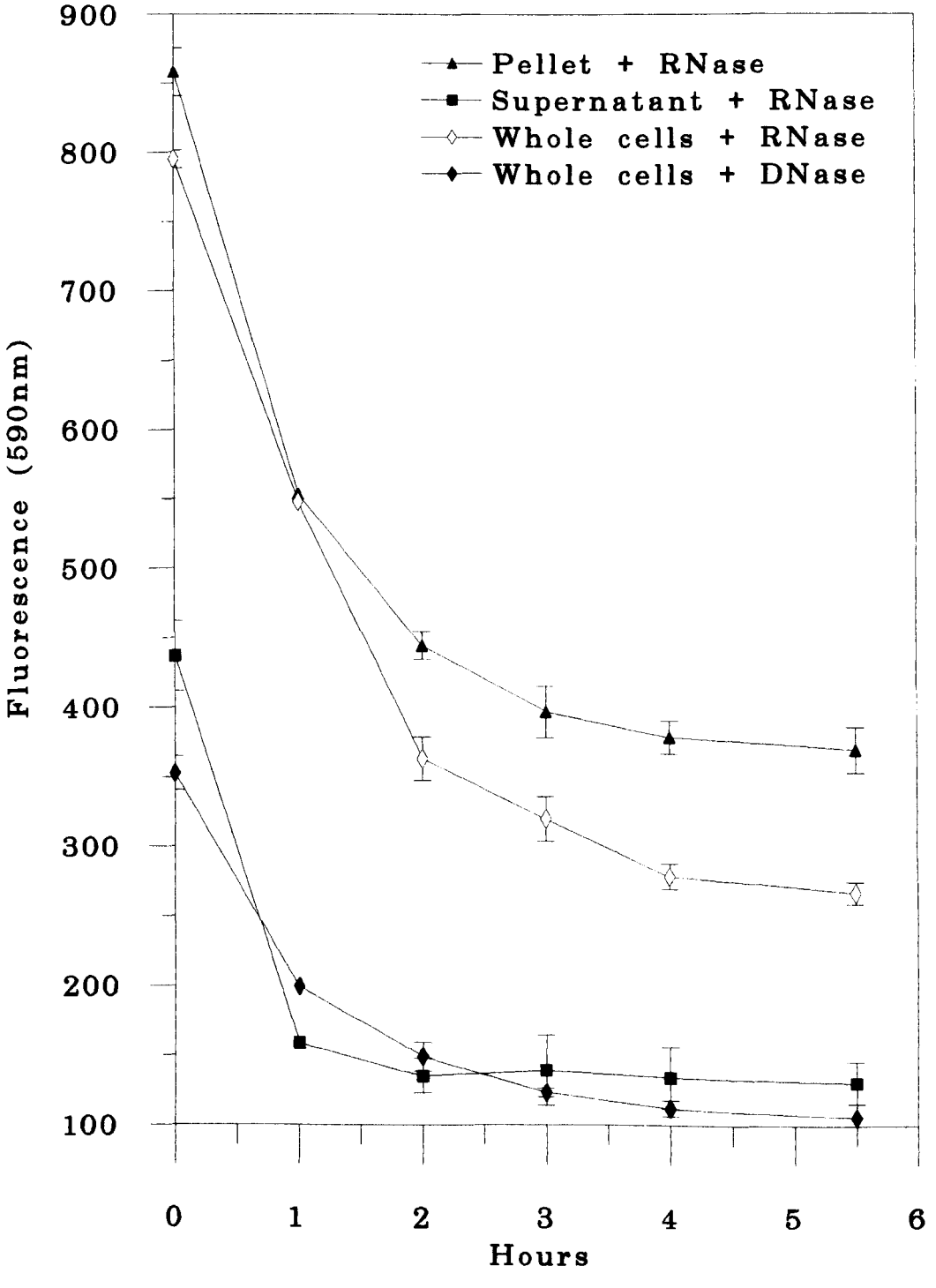
Ethidium bromide staining of *L. monocytogenes* cells gave very low levels of fluorescence. However, this could be greatly improved by incubating cell suspensions in 70% (v/v) ethanol for 1 h at 4°C prior to staining. When RNase was added to whole cells permeabilised with ethanol and the fluorescence decrease monitored with time, the untreated control preparation exhibited constant fluorescence of 792 +/-11 units, while those treated with RNase showed a rapid decrease in fluorescence within 1 h (Fig. 4.3.2.). A further gradual decline in fluorescence was observed for the duration of the experiment (up to 5.5 h) to a value of 35% of the initial total fluorescence. This suggests that most of the fluorescence is due to ethidium bromide bound to RNA, particularly in view of the differential responses of DNA and RNA noted in Fig. 4.3.1. Agarose gel electrophoresis of nucleic acid extracts showed that RNase and DNase treatment for 5.5h. removed fluorescent bands identified as RNA and DNA, respectively (Fig. 4.3.2.a). Most fluorescence disappeared when both pellet and supernatant material derived from freeze-thaw disruption were incubated with RNase. It should be noted however, that although

Figure 4.3.1. Standard Curve of Nucleic Acid Content Against Fluorescence



N.B. Determinations are means of 3 replicates. Standard deviations <10%+/- mean.

Figure 4.3.2. Fluorescence depletion due to RNase or DNase addition to whole cells and cell extracts.



freeze-thaw treatment of *L. monocytogenes* cells released a substantial amount of material that fluoresced with ethidium bromide, more than 50% of the fluorescence remained associated with cells and debrisafter 5.5h. DNase addition to whole cells permeabilised with ethanol resulted in a 45% depletion of the initial total fluorescence. The initial fluorescence was lower than that of cells suspended in RNase buffer; the fluorescence remaining after incubation with either RNase or DNase is presumably attributable largely to either DNA or RNA respectively.

Subsequent experiments showed that addition of RNase and DNase together to whole cells resulted in greater decrease in fluorescence than that due to RNase or DNase alone, but the fluorescence remaining after 5.5h was significantly greater than controls consisting of DNase buffer and ethidium bromide stain only.

4.3.2. Release of nucleic acids by physical disruption of *L. monocytogenes* cells.

1). Freeze-Thawing The data presented in Table 4.3.2. show that prior fixation of the cell pellet in ethanol for 1h enhanced the overall fluorescence signal, and also the fluorescence associated with the supernatant. Enhancement was also achieved when ethanol-fixation was carried out after freeze-thaw lysis, but in this case very little fluorescence was associated with the supernatant. When the number of freeze-thaw cycles was varied, there was a significantly greater quantity of fluorescence released, in both the total and proportion associated with the supernatant fraction, after one cycle of treatment.

2). Ultrasonication. In general, it can be observed that ultrasonication is of poor reproducibility in fluorometric determinations (Table 4.3.2.). Prior ethanol-fixation significantly suppressed the proportion of fluorescence contained in the supernatant compared with ultrasonication of unfixed cell samples. However, the total fluorescence of fixed samples was greater than that in any unfixed samples. When the number, and

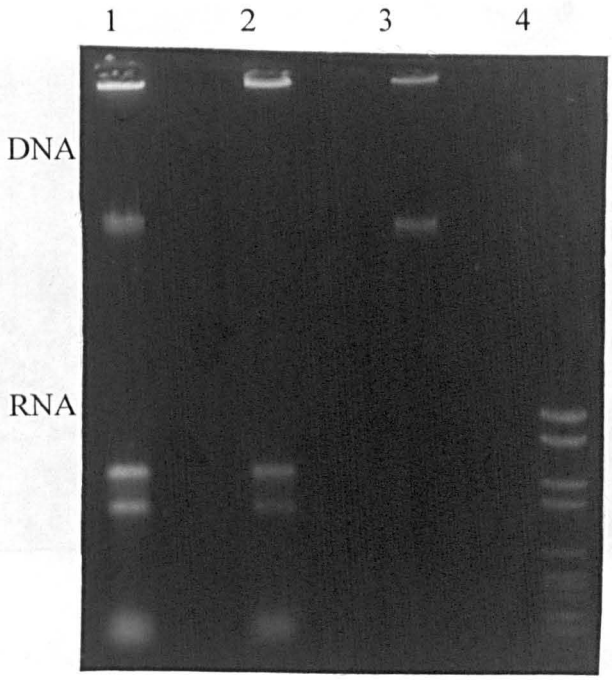


Figure 4.3.2.a. Agarose gel separation of 8µl *L. monocytogenes* nucleic acids degraded with either RNase (lane 2) or DNase (lane 3) for 5.5h. at 37°C. 10µl *L. monocytogenes* nucleic acids incubated without enzyme for 5.5h. at 37°C (lane 1). DNA molecular weight marker VI (lane 4).

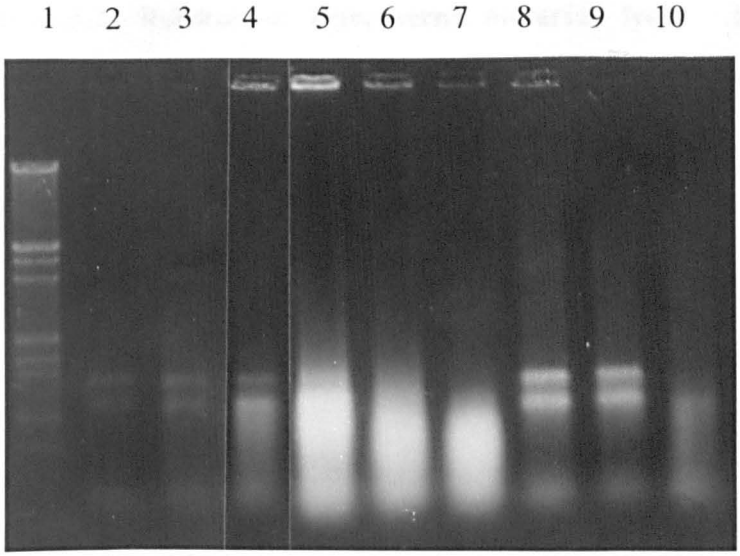


Figure 4.3.3.. Agarose gel separation of *L. monocytogenes* nucleic acids obtained by the following procedures:-

Lane 1. 2µl Molecular weight marker III (Boehringer Mannheim 528552); 5 second ultrasonication treatment for 1 cycle (lane 2), 3 cycles (lane 3) and 5 cycles (lane 4); 30 second ultrasonication for 1 cycle (lane 5), 3 cycles (lane 6) and 5 cycles (lane 7); freeze-thaw lysis for 1 cycle (lane 8), 3 cycles (lane 9) and 5 cycles (lane 10).

Table 4.3.2. Release of fluorescent material from *Listeria monocytogenes* cell preparations after application of physical disruption methods.

Method of Disruption*	Prior ethanol fixation (+/-)†	Mean Emission 590nm	Standard Deviation (+/-)‡	% Total Fluorescence in Supernatant§
Freeze thaw x 3[a]	+	342	7	40
Freeze thaw x 3	–	118	6	5
Freeze thaw x 3 post ethanol fix	–	50	7	14
Freeze thaw x 1	+	1041	40	61
Freeze thaw x 3[a]	+	968	7	53
Freeze thaw x 5	+	700	21	46
Ultrasonicationx5	+	857	19	48
	–	622	13	77
30seconds on, x1	–	413	30	42
30seconds on, x3	–	579	76	63
30seconds on, x5	–	643	19	73
5 seconds on, x1	–	481	28	61
5 seconds on, x3	–	621	76	63
5 seconds on, x5	–	624	45	64

N.B. Determinations are the means of 3 replicates. * =3X, 5X etc. refers to the number of cycles of a specific treatment. † = prior treatment for one hour at 4°C in 1ml 70 % v/v ethanol. ‡ = standard deviation of emission at 590nm. § = % total fluorescence: quantity of fluorescence associated with supernatant fraction after specific treatment expressed as a percentage of the sum of pellet and supernatant fluorescence. a = different cell preparations.

duration of ultrasonication cycles was varied, the data indicated that one cycle released significantly less fluorescence associated with pellet and supernatant than after 3 or 5 ultrasonication cycles. Furthermore, agarose gel visualisation of ultrasonicated extracts, (Fig. 4.3.3.) demonstrated that the appearance of the nucleic acid bands varied considerably with the number and duration of cycles despite high fluorescence values for sonicated pellet and supernatant material (Table 4.3.2.).

4.3.3. Release of nucleic acids by enzymatic lysis of *L. monocytogenes* cells.

1). Lysozyme. A comparison of ethanol-fixed and unfixed samples showed a significant difference between the samples in terms of the percentage fluorescence associated with the fractions (Table 4.3.3.). The unfixed sample did show a significant increase in fluorescence associated with the supernatant compared to the fixed sample.

2). Mutanolysin. When the time of incubation in the presence of mutanolysin was varied and the fractions analysed fluorometrically, there was no significant difference in total fluorescence, quantity associated with the fractions or reproducibility. Fig. 4.3.4. shows that lysis for 15 min. produced clear bands of rRNA and DNA, with very little degradation when extracts were visualised on agarose gels.

3). Achromopeptidase. The largest amount of fluorescence was released as a total and into the supernatant after 90 min incubation, (Table 4.3.3., row 3). However, there was very little difference between the 90 and 120 min treatments, or the reproducibility of the fluorescence released into the fractions with the exception of the 2h treatment. When extracts were visualised, only DNA bands could be observed (Fig. 4.3.5.) with the exception of the 15 minute treatment.

4). Proteinase K. In preliminary investigations, it was found that the suggested Proteinase K buffer raised fluorescence considerably and so the values shown in Table

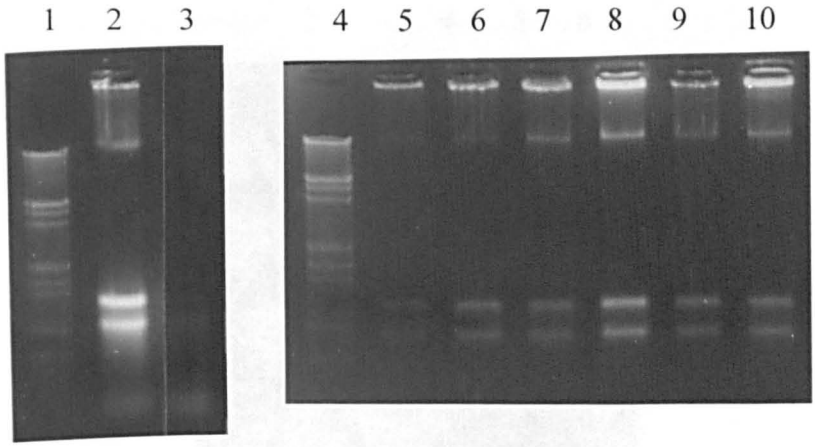


Figure 4.3.4. Agarose gel separation of *L. monocytogenes* nucleic acids obtained by the following procedures:-

Lane 1. 2µl Molecular weight marker III (Boehringer Mannheim 528552)

Lane 2. Mutanolysin 60 min at 37°C Pre-treated 1h. in 70% (v/v) ethanol at 4°C.

Lane 3. Mutanolysin 60 minutes at 37°C No pre-treatment.

Lane 4. DNA Marker III (see lane 1)

Lane 5-10. Proteinase K treatment for 15, 30, 45, 60, 90 and 120 minutes at 55°C. respectivley

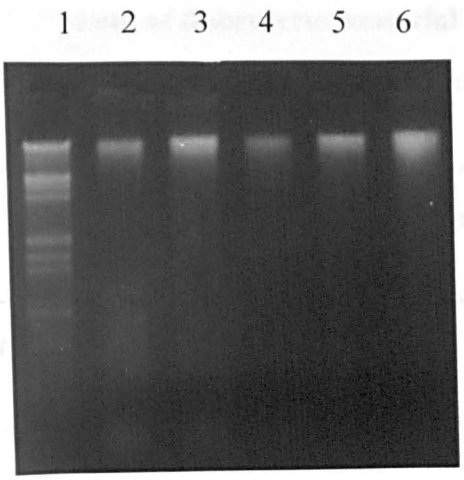


Figure 4.3.5. Agarose gel separation of *L. monocytogenes* nucleic acids obtained by the following procedures:-

Lane 1. 2µl Molecular weight marker III (Boehringer Mannheim 528552)

Lanes 2-6. Achromopeptidase treatment for 15, 30, 60, 90 and 120 minutes at 37°C respectively.

Table 4.3.3. Release of fluorescent material from *L. monocytogenes* cell preparations after application of enzymatic disruption methods.

Method of Disruption	Prior ethanol fixation (+/-)*	Mean Emission 590nm	Standard Deviation (+/-)†	% Total Fluorescence in Supernatant‡
Lyzozyme 60min,	+	31	7	4
37°C	–	44	3	5
Mutanolysin 37°C				
5 min	+	152	9	31
15 min	+	150	8	31
30 min	+	146	6	29
60min	+	114	7	24
Achromopeptidase				
60min 37°C	+	253	13	37
90min	+	363	17	44
120min	+	340	26	43
Proteinase K 65°C				
30 min	+	518	19	41
60min	+	663	16	46
90min	+	669	27	45
120min	+	496	11	36

N.B. Determinations are the means of 3 replicates. * = prior treatment for one hour at 4°C in 1ml 70 % v/v ethanol. † = standard deviation of emission at 590nm. ‡ = % total fluorescence: quantity of fluorescence associated with supernatant fraction after specific treatment expressed as a percentage of the sum of pellet and supernatant fluorescence.

4.3.3. have been corrected for background fluorescence. Incubation in the presence of this enzyme released a large amount of fluorescent material (ca. 40%) into the supernatant for all of the treatment times applied. The standard deviations reflect a reproducibility that is similar to that found for achromopeptidase, but much greater than that for the other two enzymes studied. The data showed that incubation for 90 min in the presence of this enzyme released significantly more fluorescence than the other times studied. When extracts were visualised, good banding patterns both of rRNA and DNA were observed (Fig. 4.3.4.)

4.4.

Discussion

When suspensions of whole cells were permeabilised with ethanol and treated with RNase, a rapid and substantial decay in fluorescence was observed (Fig. 4.3.2.). In controls without ethanol treatment, minimal fluorescence was detected, while in permeabilised cells, the ethidium bromide gave a strong signal that was stable over time. The decrease in fluorescence due to RNase was 65% over 330 min. (Fig. 4.3.2.) which, assuming total RNA degradation, gave a value of 5.35 μg RNA per 10^9 cells. The decrease in fluorescence due to DNase was 45% over 330min. which, assuming total DNA degradation, gives a value of 5.8 μg DNA per 10^9 cells. Incubation for 330 min was sufficient to degrade all RNA or DNA present (Fig. 4.3.2.a) in nucleic acid extracts. Cells were harvested from a late exponential/stationary phase culture, and these values for RNA and DNA content are in general agreement with data published for other bacterial species (Gausing 1977; Kemp *et. al.*, 1993; Kerkhof and Ward 1993; Kjellgaard and Kurland 1963; Moyer *et. al.*, 1990; Rosset *et. al.*, 1966). RNase treatments have been used in this way to calculate RNA/DNA ratios in bacteria (Kemp *et. al.*, 1993; Kerkhof and Ward, 1993) by referring to standard curves and slope ratios for RNA and DNA fluorescence published by LePecq & Paoletti (1966) using calf thymus DNA and RNA derived from yeast and rat liver. In this study, standard curves were prepared with *Listeria monocytogenes* DNA and *E. coli* ribosomal RNA and the slope ratio found to be 3.9. This was significantly different to that (2.82) reported by LePecq and Paoletti (1966), and subsequent studies (Chapter 5) analyse RNA/DNA ratios of *L. monocytogenes*. Therefore, use of *L. monocytogenes* nucleic acids to determine the relationship between nucleic acid concentration and fluoresence is of increased relevance.

Physical disruption techniques were found, with the exception of proteinase K digestion for 90min, to release significantly more total fluorescent material into the supernatant than enzymatic methods. However, when visualised by gel electrophoresis,

there was more degradation evident with nucleic acid extracts from cells lysed by freeze-thaw treatment or ultrasonication than from enzymatically lysed cells. The stained bands produced by enzymatic lysis were less intense but more defined (Figs. 4.3.4. and 4.3.5.). These findings imply that although substantial quantities of fluorescent material are released from the cells by physical disruption, the structural integrity of DNA in particular, has not been preserved.

Although highest yields of fluorescence released into supernatants were best obtained by repeated ultrasonication, this resulted in degradation of nucleic acid, visible on agarose gels as a smear (Fig. 4.3.3. lanes 4-6). This could be rectified by using only a single cycle of ultrasonication treatment but with a concomitant and significant decrease in fluorescence yield. However, while the integrity of RNA was retained in this way, even single cycles of ultrasonication did not yield intact bands of DNA. The objective of releasing large amounts of both RNA and DNA in undegraded form cannot be met by the application of ultrasonication to cell pellets. Ultrasonication is often used to shear DNA to prevent non-specific binding of nucleic acid probes to membranes in nucleic acid hybridisation assays (Sambrook *et. al.*, 1982). This also applies to freeze-thaw disruption of cells, although in this study a single cycle of freeze-thaw disruption released significantly greater amounts of fluorescent material into the supernatant than application of repeated cycles of treatment. These data reflect the greater structural stability of rRNA compared to DNA, and thus its resilience to the shearing effects inherent in these physical disruption methods.

The enzymatic cell lysis treatments evaluated generally gave lower yields of fluorescence than physical disruption, but reproducibility of values obtained was much improved (Table 4.3.3.). The data also showed that for *Listeria monocytogenes*, lysozyme was not effective at releasing significant amounts of nucleic acid; for example the lysozyme treatment released only 4% of the total fluorescence into the supernatant compared with 46% for the Proteinase K treatment. Lysozyme has often been used in

protocols for nucleic acid extraction from bacteria, and was the enzyme treatment used by Georgellis *et. al.*, (1992) to monitor RNA content during growth of *E. coli*. Of the other enzyme treatments, lysis for 60 min at 37°C in the presence of mutanolysin produced the most defined, and hence undegraded, bands of DNA and RNA on agarose gels (Fig. 4.3.4.). It has recently been reported that mutanolysin is suitable for releasing *L. monocytogenes* DNA from foodstuffs (Dickinson *et. al.*, 1995). However, this must be set against the much greater fluorescence yields obtained with Achromopeptidase and, particularly, Proteinase K treatments. Recently, studies have reported the use of a *Listeria*-specific recombinant phage that effectively releases 15-20μg/10^9cells of nucleic acid (Loessner *et. al.*, 1995). This has potential for *Listeria* specific, efficient nucleic acid extraction from a mixed culture.

In attempting to develop an optimised protocol for the extraction of nucleic acids from *Listeria monocytogenes*, the importance of obtaining a compromise between yield and integrity must be borne in mind. The linear relationship between fluorescence and nucleic acid concentration, and the slope ratios of DNA and RNA (Fig. 4.3.2.) are based on undegraded material and this together with the objective of producing nucleic acid for fractionation, amplification and oligonucleotide probing demands that prominence is given to minimising degradation. Achromopeptidase treatment, except after 15 min. incubation, appeared to release only DNA (Fig. 4.3.5.), the most likely explanation for which was the presence of contaminating RNase in the enzyme preparation.

Therefore, for simultaneous extraction of DNA and RNA from *Listeria monocytogenes* suitable for quantitative oligonucleotide probing, the lysis treatment used in the following studies is based on the use of Proteinase K for 90 min, which gives a high yield of fluorescence while maintaining an acceptable standard of nucleic acid structural integrity.

CHAPTER 5.

THE RELATIONSHIP BETWEEN RNA/DNA RATIOS AND THE GROWTH OF *Listeria monocytogenes*.

5.1. Aims

• To apply analytical techniques developed in chapters 3 and 4 to evaluate RNA/DNA ratios over batch growth with respect to time and nutrient availability.

5.1.1. Investigation of RNA/DNA ratios in shake flask culture.

Materials and Methods.

Strains and Growth Conditions

Conical flasks (5l volume) containing 2l TSYGB pH 7.2 were inoculated with $2.5x10^{10}$cfu of an overnight culture of *L. monocytogenes* ATCC 19111. Incubation with agitation was at 37°C and 10ml aliquots were removed at intervals. Growth was determined by measurement of optical density (1ml aliquots) at 660nm on a Pye Unicam UV-Vis spectrophotmeter, and colony counts determined on NAB agar. In addition, media pH was recorded during growth by insertion of a pH probe into an aliquot of growth media.

Analysis of cellular RNA and DNA.

Samples (6 x 1ml aliquots) of cell suspension were harvested by centrifugation at 740g for 10min. in 1.5ml eppendorf tubes and washed twice in 1ml PBS buffer pH7.2. Cell pellets were resuspended in 1ml 70% (v/v) ethanol and fixed for one hour at 4°C. Fixed cells were pelleted by centrifugation at 740g for 10min and resuspended in either RNase, DNase or lysis buffer, as appropriate. Cell samples resuspended in RNase or DNase buffer were analysed by whole cell fluorometry described above (chapter 4). Samples resuspended in lysis buffer were subjected to nucleic acid extraction with proteinase K as described above (chapter 4) and nucleic acids (12µl aliquots) visualised on 1.5% agarose gels. Triplicate samples (3µl each) of nucleic acid extracts were added to either 3ml RNase or DNase buffer, as appropriate, in 3.5ml plain-sided fluorometry cuvettes. These were equilibriated at 37°C for 12h. in

an air-tight container to minimise evaporation before recording fluoresence at 590nm. Subsequently, 3µl of either DNase free RNase or RNase free DNase were added to appropriate tubes and mixed thoroughly prior to incubation for 12h at 37°C. The decrease in fluoresence at 590nm was measured after this incubation period. The concentration of nucleic acids was calculated as described in chapter 4 and RNA/DNA ratios calculated.

Nucleic acid extracts were prepared for quantitative oligonucleotide probing by digestion of RNA or DNA, with the appropriate enzyme for 5.5h at 37°C. Nucleic acids in triplicate series were immobilised on nylon membranes with a vacuum transfer apparatus as described in chapter 2. Membranes were dried, baked and pre-hybridised using standard techniques and conditions. Hybridisation reactions were carried out overnight in the presence of either 10pM radio-labelled MV9RP2 probe (RNA analysis) or pA probe (DNA). Filters were washed for 5min. at room temperature and X-ray film exposed for 2 and 7 days prior to scanning densitometry analysis. The relationship between nucliec acid concentration and pixel intensity above background was examined by the least square method of regression analysis. Correlation coefficients were calculated from Pearson's product moment correlation coefficient (r^2).

5.2. Monitoring RNA/DNA ratios in batch fermentation.

Fermentor vessels (L.H. 501 series) containing 1.5l. TSYGB media buffered with KH_2HPO_4 ($8.5g.L^{-1}$)were prepared and maintained at 37°C, pH 6.8 for 24 h. prior to inoculation with $10^{10}cfu.ml^{-1}$ of an overnight culture of *L. monocytogenes* ATCC 19111. Constant rates of stirring, air flow and pH were maintained throughout the experiment. Samples were removed at intervals up to 456h. for determination of colony forming units, optical density at 660nm and nucleic acid content of both whole cells and nucleic acid extracts. Analytical techniques and conditions were as described above.

5.3. RNA/DNA ratios as a function of growth rate.

5.3.1. Aims.

- To investigate the relationship between experimental values of RNA/DNA ratios obtained by fluorometry and oligonucleotide probing with respect to growth rate.
- To compare the data for *L. monocytogenes* with those reported for other organisms.

5.3.2. Materials and Methods.

Media.

Modified Trivett and Meyer (1971) defined medium for *Listeria* spp. was used throughout these studies. Briefly, for 1 litre media, KH_2PO_4, (8.5g); NaH_2PO_4, (1.5g) and NH_4Cl, (0.5g) were dissolved in 500ml distilled water (solution A). NaOH, (0.24g) and nitrilotriacetic acid, (0.48g) were dissolved in 40ml warm distilled water (solution B). $MgSO_4$, (0.41g) was dissolved in 30ml distilled water (solution C). Solutions B and C were added to solution A with a further 390ml distilled water. The basal medium was autoclaved for at least 15 min (dependant on volume) at 15psi and allowed to cool. To this solution was added sterile amino acids, trace elements, vitamins and carbon source in a laminar flow cabinet. Briefly, 100X amino acid solution was prepared by dissolving L-cysteine.HCl, (10g); L-leucine, (10g); D/L-isoleucine, (20g); D/L-valine, (20g); D/L methionine, (20g); L-arginine.HCl, (20g); L-histidine.HCl, (20g) in warm distilled water at pH 8.5. Solutions were membrane filtered (0.2μm pore size) and dispensed into double autoclaved bottles. A 100X vitamin solution was prepared as follows: α-lipoic acid (5mg) was dissolved in 200ml 70% (v/v) ethanol and 2ml of this solution was added to 125ml 95% (v/v) ethanol containing 5mg biotin, 50mg thiamin.HCl and 50mg riboflavin. The solution was made up to 500ml with sterile distilled water and membrane filtered (0.2μm pore size) as above. Trace element solution (100X) comprised ($g.l^{-1}$) $CaCl_2.2H_2O$, 0.275; $ZnCl_2$

0.085; $CuCl_2$ 0.0215; $CoCl_2$, 0.03; $Na_2MoO_4.2H_2O$, 0.03 in sterile distilled water. The solution was filter sterilised (0.2μm pore size) before use.

The optimum concentration of Fe^{2+} for growth ($44mg.l^{-1}$) and the growth rate limiting concentration of glucose (<15mM) were found in preliminary studies. Briefly, 10ml amounts of Trivett and Meyer media had various concentrations of ferric citrate added (0-1ml of a 100X solution comprising $8.8g.l^{-1}$ ferric citrate in sterile distilled water). This was inoculated with *L. monocytogenes* and incubated for 24h at 30°C. Growth was assessed by measurement of optical density at 660nm after 11 and 24h. Culture purity was confirmed by spreading on NAB plates. Growth rate limiting glucose concentration was determined by the addition of a range of glucose concentrations (0.35-52 mM) to Trivett and Meyer medium. Flasks were inoculated with *L. monocytogenes* and growth assesssed at intervals by measurement of optical density at 660nm.

In order to vary growth rate, 500ml volumes of Trivett and Meyer media in conical flasks were supplemented with either 0, 0.151, 0.757, 1.51, 2.5, 7.55, 22.7, or 37.8mmoles glucose. Sterility was checked by incubation for 24h at 30°C. An inoculum was grown overnight at 30°C in TSYGB and harvested by centrifugation at 740g for 15 min. Cell preparations were washed twice in 20ml PBS buffer (pH 7.4) and resuspended in a final volume of PBS buffer prior to inoculation.

Flasks were inoculated with 10^7 cfu. *L. monocytogenes* ATCC 19111 and incubated with agitation at 30°C. Samples (8ml) were removed aseptically at intervals for measurement of growth (O.D. 660nm) and nucleic acid concentrations. Colony forming units were measured on NAB plates when optical density readings were indicative of exponential growth phase. This permitted the calculation of growth rate between sampling points. Nucleic acid concentrations were assessed when cultures were in the exponential phase of growth (13 h). Cell samples (1ml volume in triplicate) were pelleted by centrifugation at 740g for 7min and washed twice in 1ml PBS buffer (pH 7.4). Samples were fixed in 70% (v/v) ethanol prior to the fluorometric measurement of nucleic acid in whole cells and extraction of nucleic

acids for further quantitative analysis. Measurements of nucleic acid extracts were obtained by oligonucleotide probing and fluorometry and followed identical procedures and conditions to those described earlier.

5.4. RNA/DNA ratios in *L. monocytogenes* after sub-lethal injury.

5.4.1. Aims.

- To examine the effect of sub-lethal injury by either acid or temperature treatments on survival and recovery of *L. monocytogenes* and of nucleic acid ratios.

- To examine the effect of cultural history on the response of *L. monocytogenes* to thermal injury.

5.4.2. Materials and Methods.

Acid shocking experiments.

Batch culture fermentations containing 2l. of Trivett and Meyer (1971) defined medium with excess glucose (6.25g.l^{-1}) were prepared and maintained for 24 h. at 30° C, pH 6.8 with aeration to verify sterilty. Fermentors were inoculated with 10^{11} cfu *L. monocytogenes* ATCC 19111 cultured overnight on TSYGB at 30°C and prepared as described earlier.

In an initial experiment the culture was grown to stationary phase (O.D.660nm = 0.93 at 24 h.). Sufficient 1M HCl was added to the culture to attain pH 3.0 which was maintained for 30min. Acid was pumped manually into the fermentor to achieve a rapid pH descent. Following acid exposure, 5M NaOH was quickly added to return the culture to pH 6.8. Samples (8 ml) were removed at 5, 7.5 and 20 h. growth prior to

addition of acid. After acid addition, samples were removed after fermentor pH had been raised to pH 6.8 following 30min acid exposure (20.5 h post-inoculation), and after a further 1, 2, 4.5 and 24 h.

Exposure of a mid-exponential phase culture to HCl and monitoring subsequent RNA/DNA levels was also attempted. Briefly, 2l batch fermentations of Trivett and Meyer (1971) media were set up and sterility checked at 30°C, pH 6.8 with agitation and constant air flow. The fermentor was inoculated with 10^{11} cfu *L. monoyctogenes* and growth monitored by measurement of optical densitity until mid-exponential phase was attained (O.D. 660nm = 0.270, 8 h.). 1M HCl was added to the culture, sufficient to lower pH to 3.0. This pH was maintained for 30min before 5M NaOH was added to raise the culture to pH 6.8. Samples (8ml) were removed for analysis immediately prior to acid addition (t = 0), and following 30min exposure to HCl, when the culture had been returned to pH 6.8 (t = 0.5 h.). Further samples were removed at 2, 4, 6 and 24 h. after exposure to HCl.

Measurement of growth in both experiments was achieved by optical density and colony forming units per ml. culture. Nucleic acid concentrations were assessed by fluorometry of whole cells and nucleic acid extracts derived from 1ml samples of culture. Immobilised nucleic acids were subject to quantitative oligonucleotide probing with analysis by scanning densitometry. Procedures and conditions for fluorometry and nucleic acid hybridisation were identical to those employed in earlier studies.

5.5. Effect of Thermal shocking on *L. monocytogenes* growth.

5.5.1. Materials and Methods.

Trivett and Meyer media (TM) and Trivett and Meyer media supplemented with 1%(v/v) pyruvate solution (TMP, added as a filter sterilised solution post-

autoclaving); and TSYGB media, TSYGB supplemented with 1% (v/v) pyruate solution (TSYGBP; 1L each media) was added into duplicate 2l conical flasks. Flasks were incubated with agitation for 24 h. at 30°C to ensure sterilty.

Inocula were prepared as follows. 100ml conical flasks containing TSYGB were inoculated with a loopful of *L. monocytogenes* ATCC 19111 culture and incubated for 12h., with agitiation at both 20°C and 40°C. Cells were harvested by centrifugation at 740g for 15 min. prior to washing pellets twice in PBS buffer (pH 7.4) held at the appropriate growth temperature.

Cells samples, cultured at both 20°C and 40°C, were subjected to heat shocking by placing inocula in pre-warmed sterile centrifuge tubes (100ml volume) previously equilibriated in a water bath at 58°C for 10min. This temperature has been previously shown to cause *L. monocytogenes* injury (Stephens and Jones 1993). Cells (10^{10} cfu) from cultures at both growth temperatures were used to inoculate each of the media formulations. Colony forming units were evaluated prior to, and immediately following, heat shocking.

Growth was monitored at 1, 3, 5, 9, 11, 24 h. after inoculation. Samples (8ml) were aseptically removed and used to monitor optical density (660nm) colony forming units per ml., nucleic acid content by fluorometric analysis of whole cells and nucleic acid extracts, and quantitative oligonucleotide probing of nucleic acid extracts. Procedures and conditions followed for these assessments were identical to those used for earlier studies.

5.6. RESULTS

5.6.1. RNA/DNA ratios during shake flask culture.

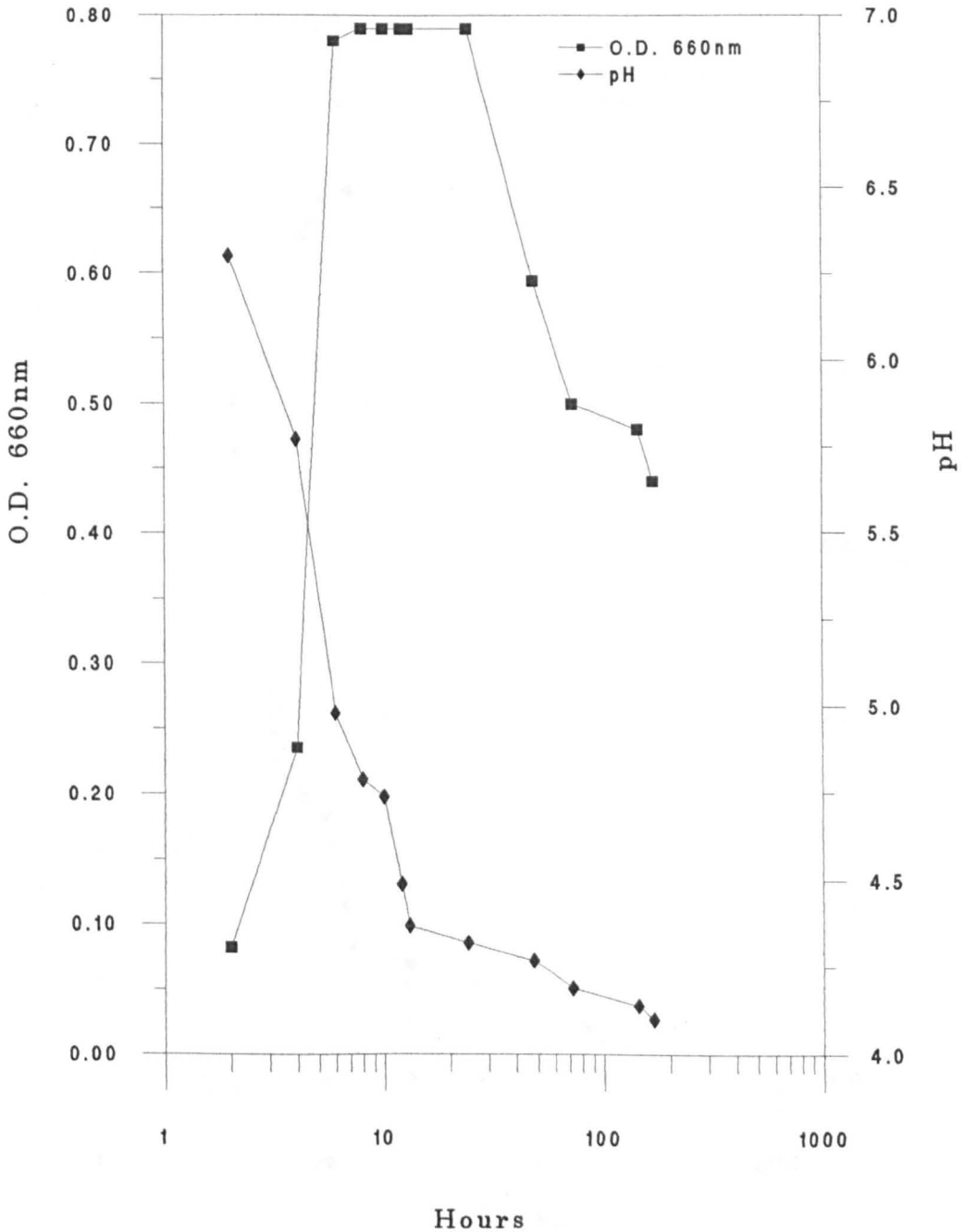
Growth of *L. monocytogenes* in a complex unbuffered medium was monitored over time. A predictable relationship between optical density and time is shown in Fig. 5.6.1.1.a. The number of colony forming units observed increased until 13h. after inoculation and rapidly decreased thereafter. Subsequent samples (from 24h.) did not produce any colony forming units on the medium used (Fig. 5.61.1.b.).

Nucleic acid extracts visualised by agarose gel electrophoresis are presented in Fig. 5.6.1.2. A clear increase in nucleic acid band intensity is seen from the first sample examined (6h. after inoculation) until 24 h. incubation. From this sample point until the end of the experiment (148h.) nucleic acids are visualised, although significant degradation can be seen.

Autoradiographs produced with titrated nucleic acid preparations and experimental nucleic acid samples were subjected to oligonucleotide probing and analysis by scanning densitometry. Graphs of standard nucleic acid concentration against pixel sum above background were used to generate standard curves for both RNA and DNA (Fig. 5.6.1.3. and 5.6.1.4. respectively). The relationship between nucleic acid concentration and pixel intensity above background was examined by the least square method of regression analysis. Equations describing the line of best fit were $y = 147.22 + 181.98x - 1.78x^2$; $r^2 = 0.98$ (DNA) and $y = -4.31 + 35.45x + 0.50x^2$; $r^2 = 0.99$ (RNA). Experimental values of RNA and DNA concentrations from samples were calculated from these equations and used to determine RNA/DNA ratios (Fig. 5.6.1.6.).

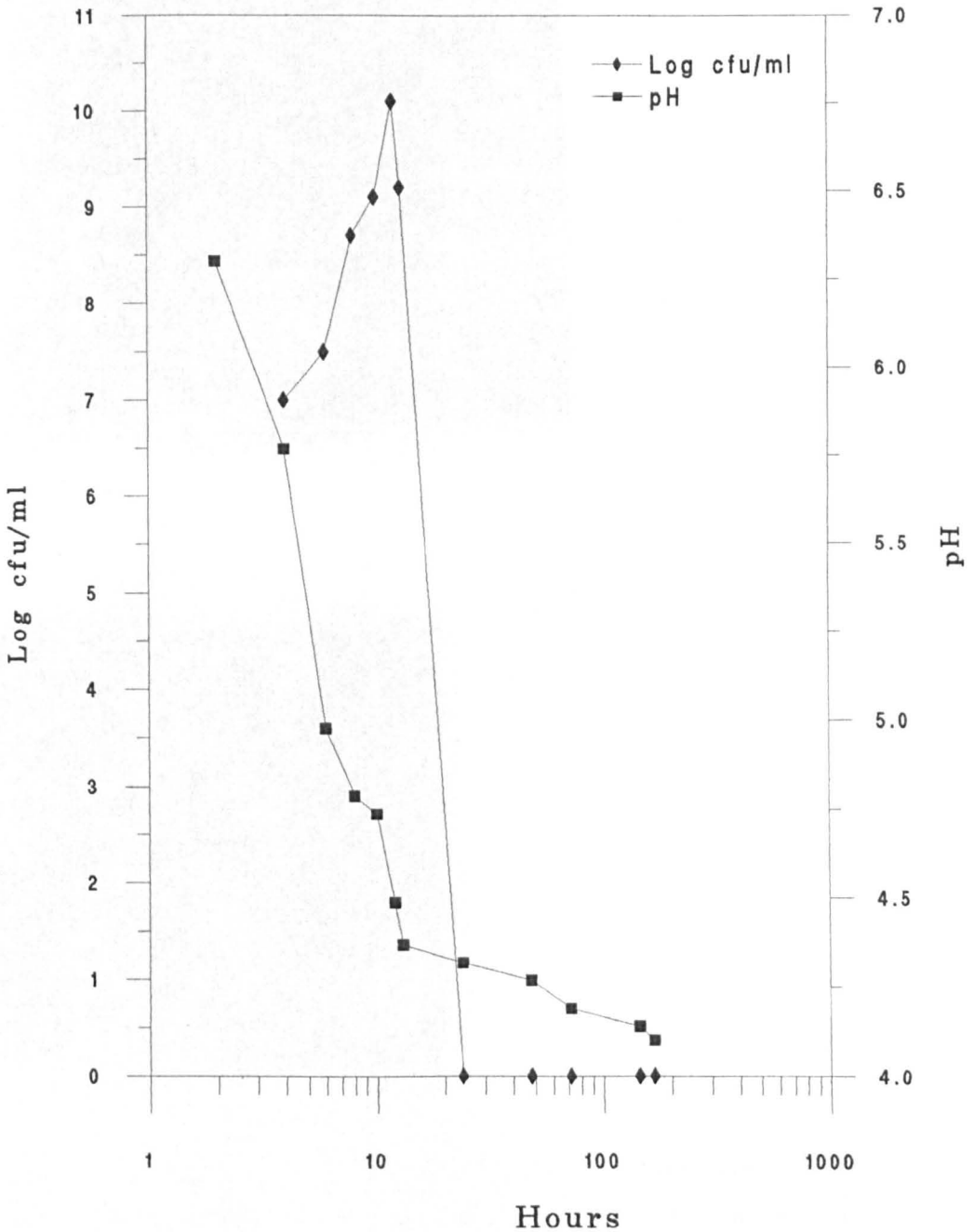
Comparison of RNA/DNA ratios determined by fluorometry and oligonucleotide probing show a similar relationship over the duration of the experiment (Fig. 5.6.1.5. and 5.6.1.6). RNA/DNA ratios rose rapidly until late exponential, or

Figure 5.6.1.1a. Graph of L. monocytogenes growth (optical density at 660nm) and pH in an unbuffered complex media.

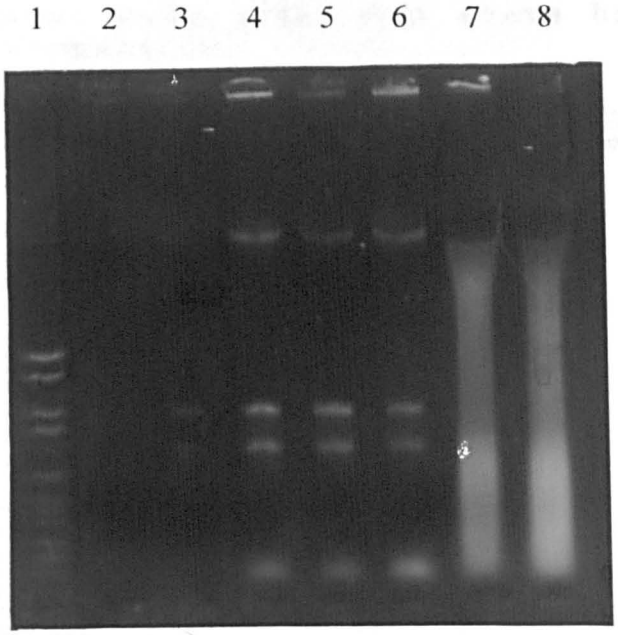


N.B. Points are the result of triplicate means. Standard deviations <10% +/-mean.

Figure 5.6.1.1.b. L. monocytogenes growth (cfu/ml) and culture pH in an unbuffered complex media.



N.B. Determinations are triplicate means.
Standard Deviation < +/-10% mean.



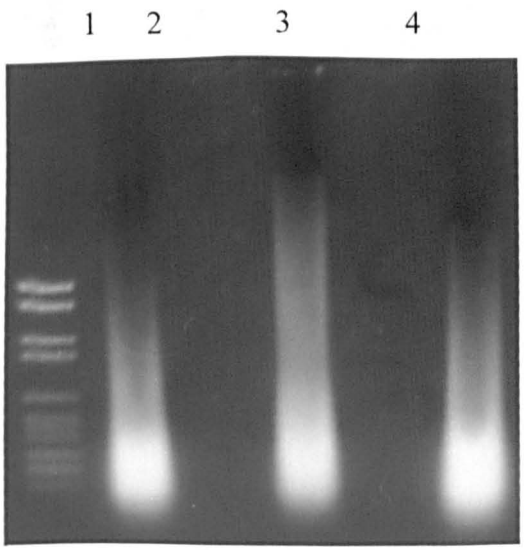


Figure 5.6.1.2. Nucleic acid extracts obtained from 1ml broth from samples removed during shake flask culture. From Left to Right: (upper) molecular weight marker VI; 4, 8, 10, 12, 13, 24, 48 h. incubation. (lower) molecular weight marker VI, 72, 120 and 144h. incubation.

Figure 5.6.1.3. Calibration curve of RNA concentration against mean pixel sum above background for shake flask experiment.

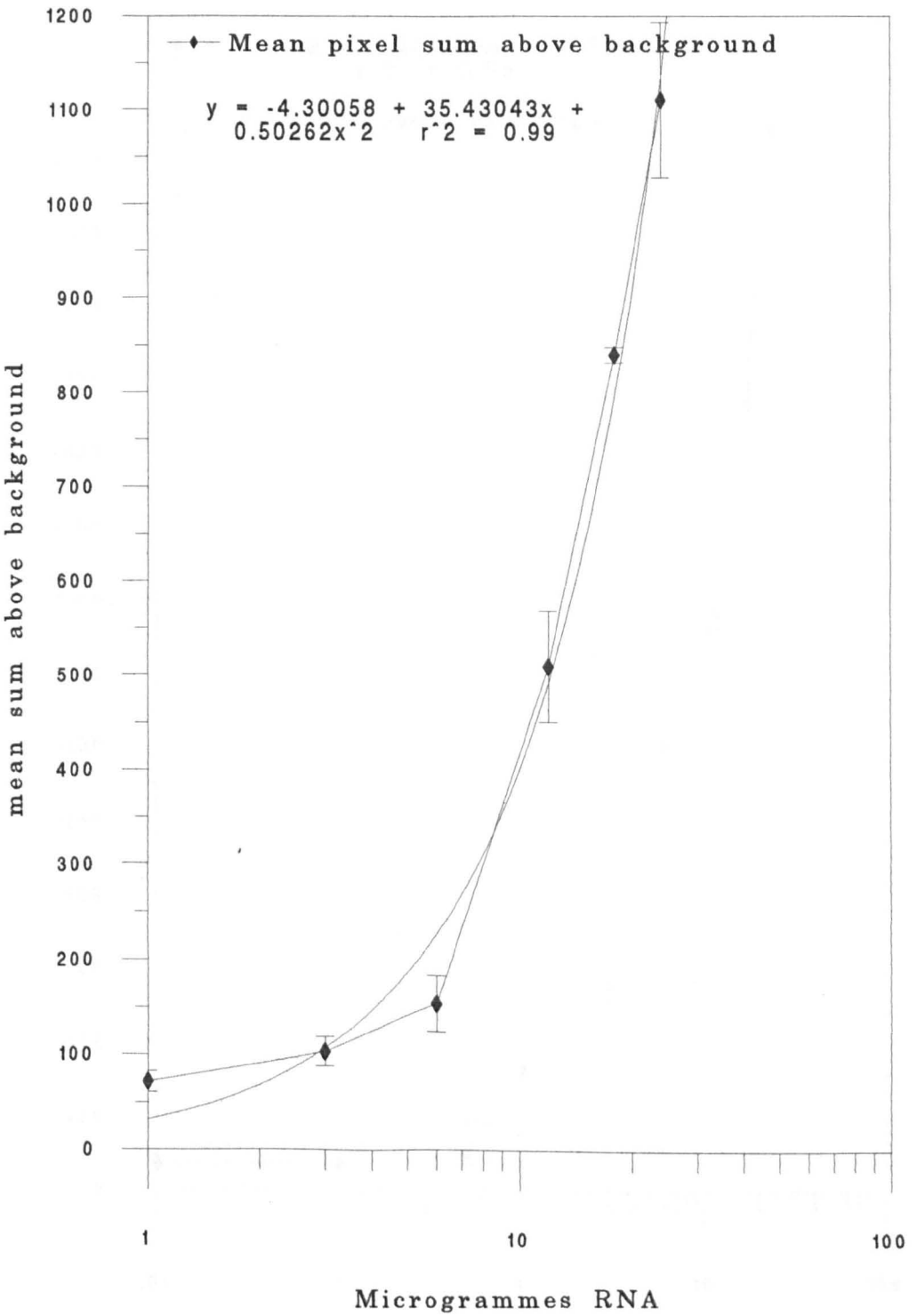


Figure 5.6.1.4. Graph of DNA concentration against pixel intensity mean sum above background for shake flask experiment.

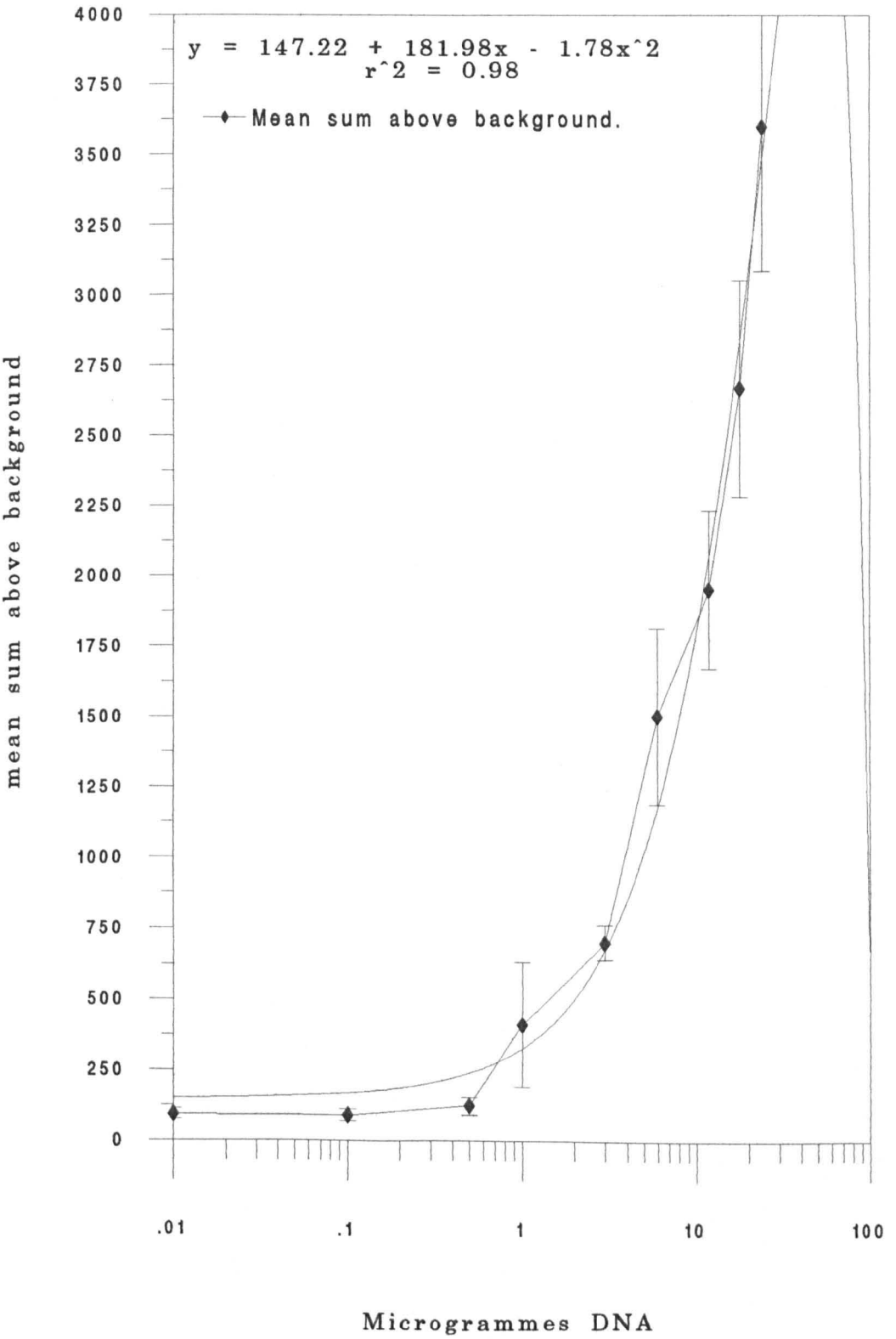


Figure 5.6.1.4. Graph of DNA concentration against pixel intensity mean sum above background for shake flask experiment.

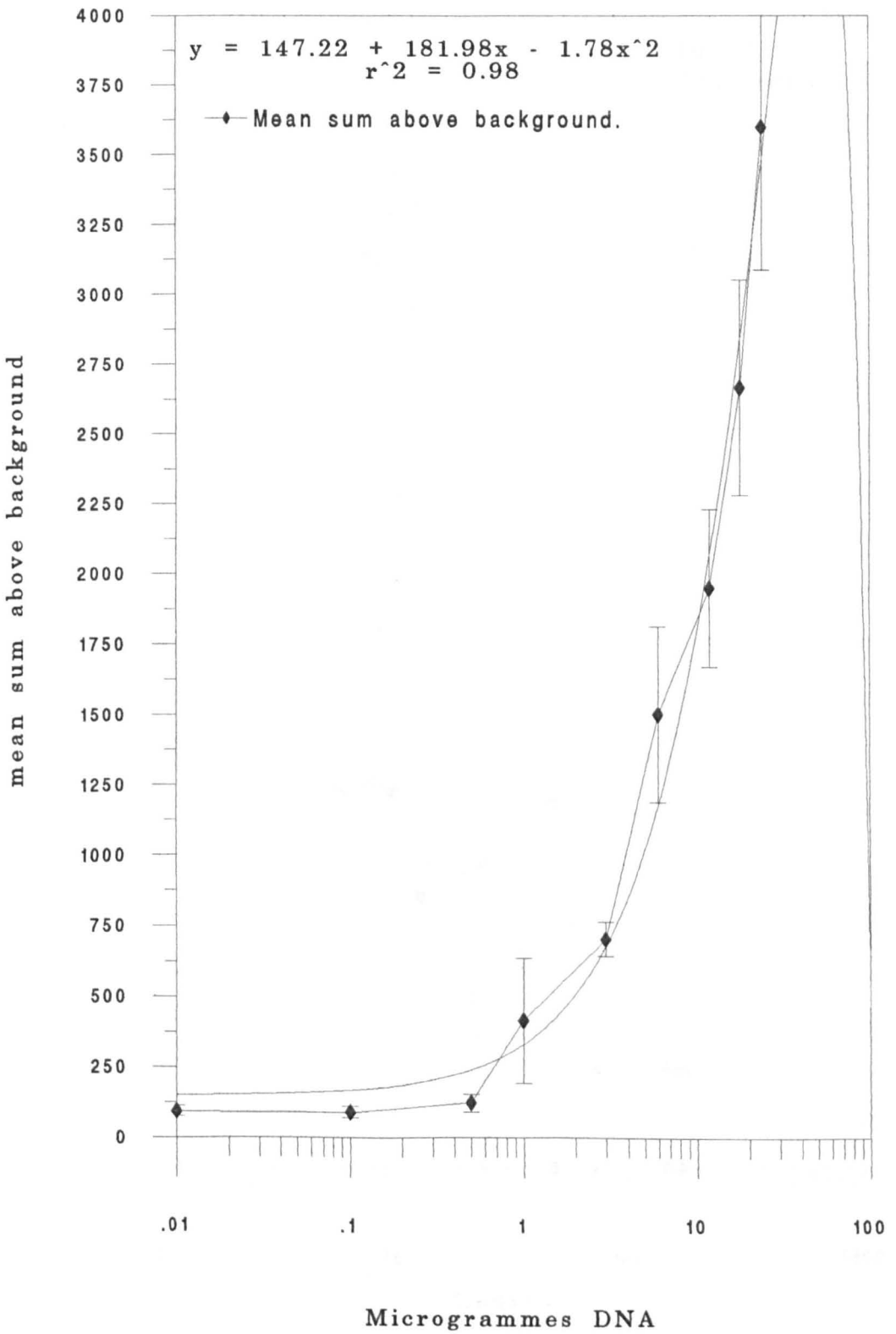


Figure 5.6.1.5. Shake flask culture of L. monocytogenes and RNA/DNA ratios measured by fluorometry of nucleic acid extracts.

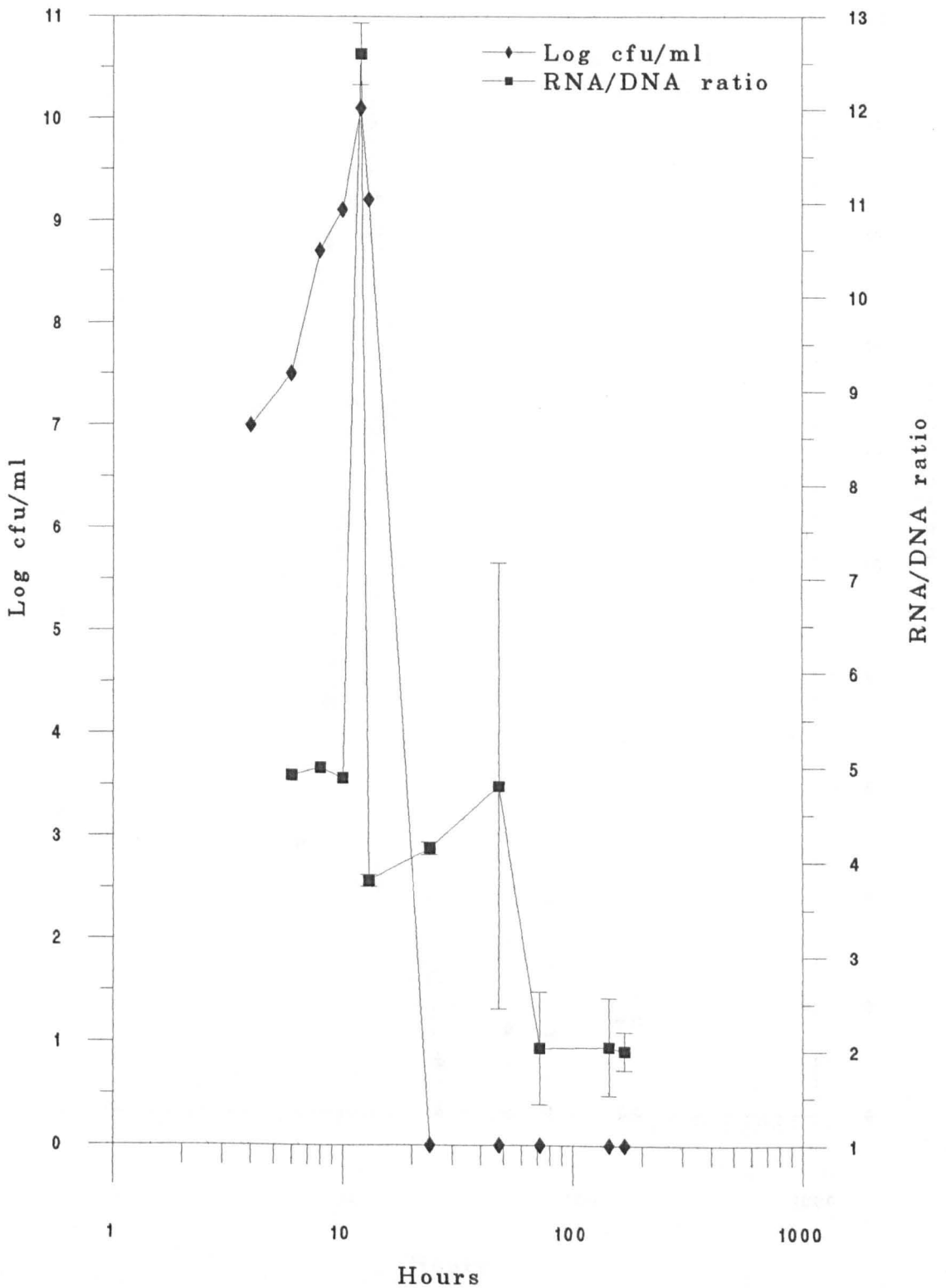
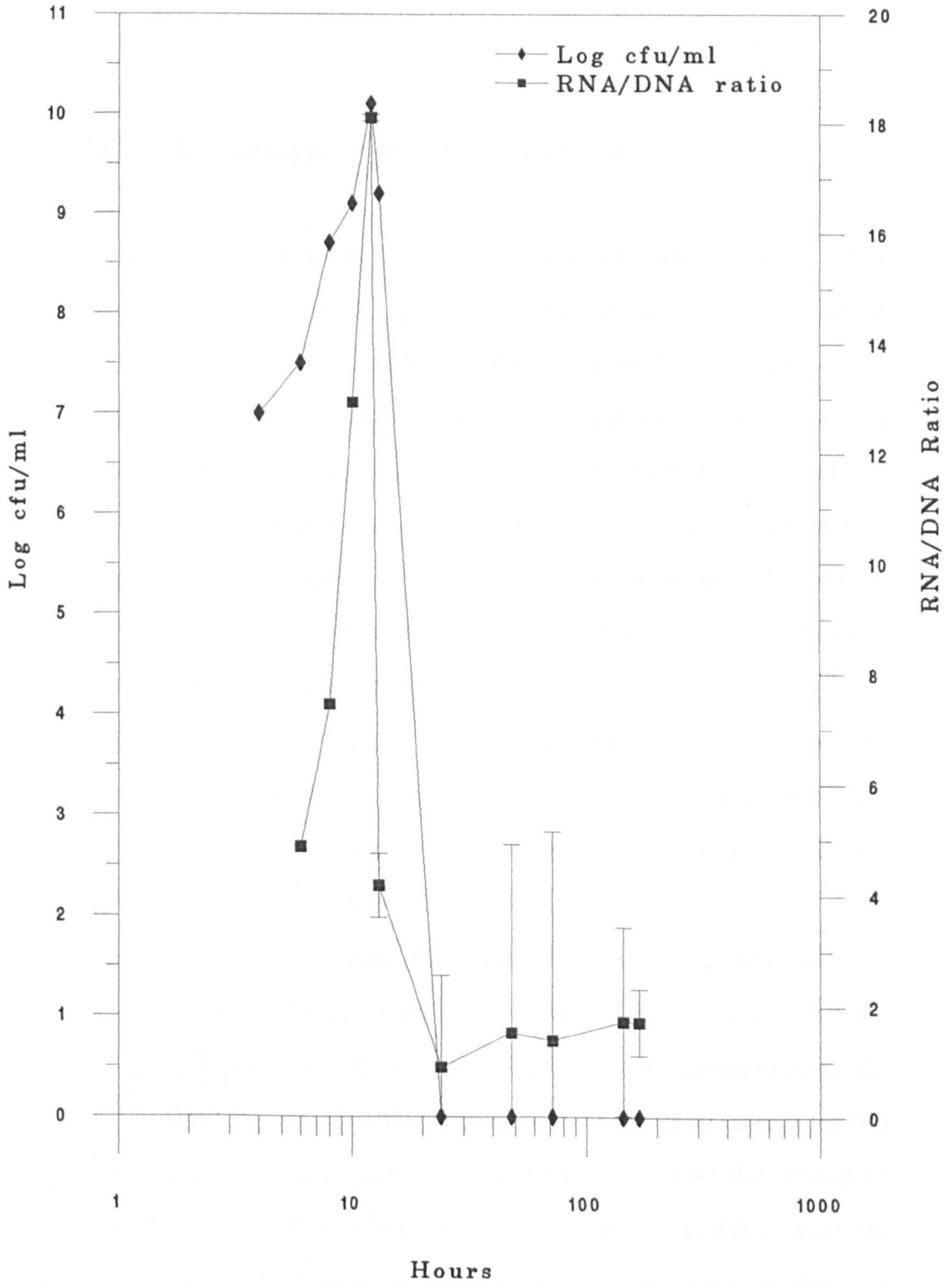


Figure 5.6.1.6. Growth of L. monocytogenes in shake flask (cfu/ml.) and RNA /DNA ratios monitored by oligonucleotide probing.



early stationary phase of culture. Subsequently, nucleic acid ratios fell rapidly, and further decreases were not statisically significant.

5.6.2. RNA/DNA ratios during pH controlled batch growth.

L. monocytogenes was grown in complex media in a pH controlled batch culture over a period of 17 days. Cell growth was monitored by determination of colony forming units and optical density at 660nm. Nucleic acids extracts were visualised by agarose gel electrophoresis (Fig. 5.6.2.1.) and showed an increase in nucleic acid band intensity with culture time until 12h. Subsequent extracts showed a constant intensity which decreases after 24h. Nucleic acid ratios were measured by fluorometry of whole cells, nucleic acid extracts and oligonucleotide probing techniques. The relationship over time of optical density and colony counts showed an expected relationship for batch growth (Fig. 5.6.2.2.).

Analysis of RNA and DNA content of whole cells and nucleic acid extracts by fluorometry showed a rapid increase in RNA content during exponential growth (Figs. 5.6.2.3. and 5.6.2.4., respectively). This was followed by a substantial decrease during stationary phase; any further decreases were gradual.

Calculation of RNA/DNA ratios by oligonucleotide probing were achieved using scanning densitometry. Relationships between standard nucleic acid (both RNA and DNA) concentration and pixel intensity above background are shown in Appendix 5.6.1. and 5.6.2. respectively. Regression analysis of the data by least squares method produced formulae of $y = 110.17 + 151.39x + 0.23516x^2$; $r^2 = 0.97$ (DNA) and $y = 158.30 \times 10^{(0.0881x)}$; $r^2 = 0.99$ (RNA). RNA/DNA ratios calculated from the oligonucleotide probing data *L. monocytogenes* colony forming units are shown in Fig. 5.6.2.5. Nucleic acid ratio increases from 2 h. reaching a maximum value immediatley prior to the end of exponential phase (8 h.).

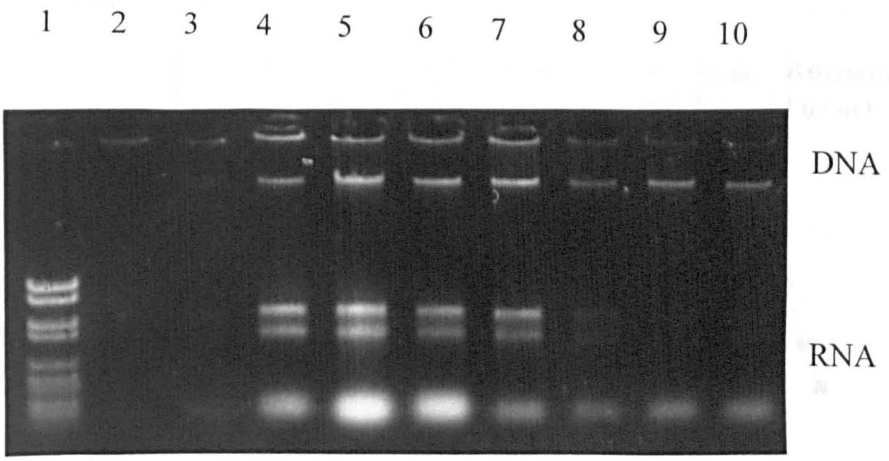
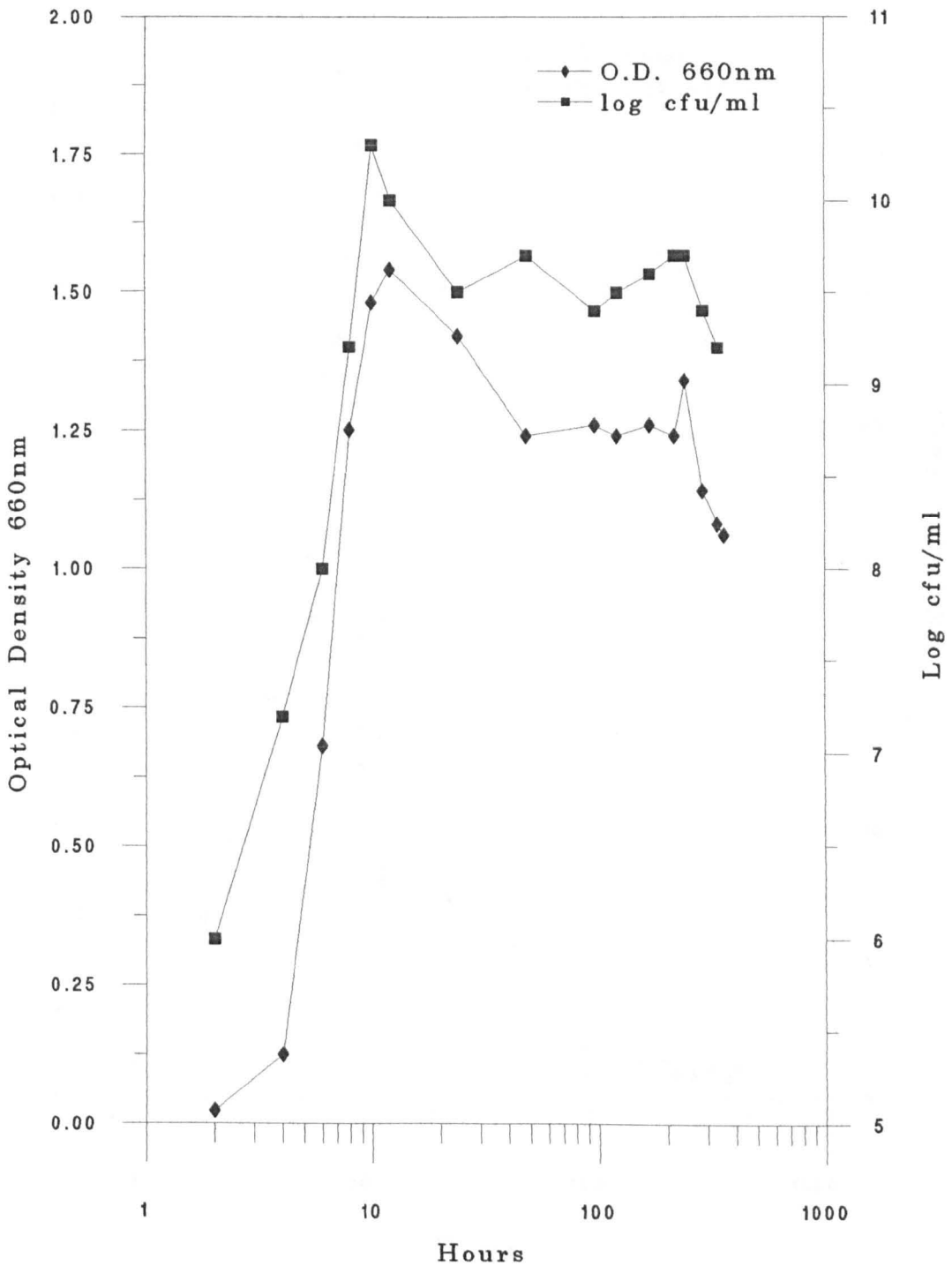


Figure 5.6.2.1. Nucleic acid extracts obtained from samples taken from pH controlled batch culture. Left to Right: molecular weight marker VI, 2, 4, 6, 8, 10, 12, 24, 48, 96h. incubation.

Figure 5.6.2.2. L. monocytogenes growth in pH controlled batch culture.



N.B. Values are the result of triplicate means.

Figure 5.6.2.3. Growth of L. monocytogenes (cfu/ml) and RNA/DNA ratios measured by whole cell fluorometry in pH controlled batch culture.

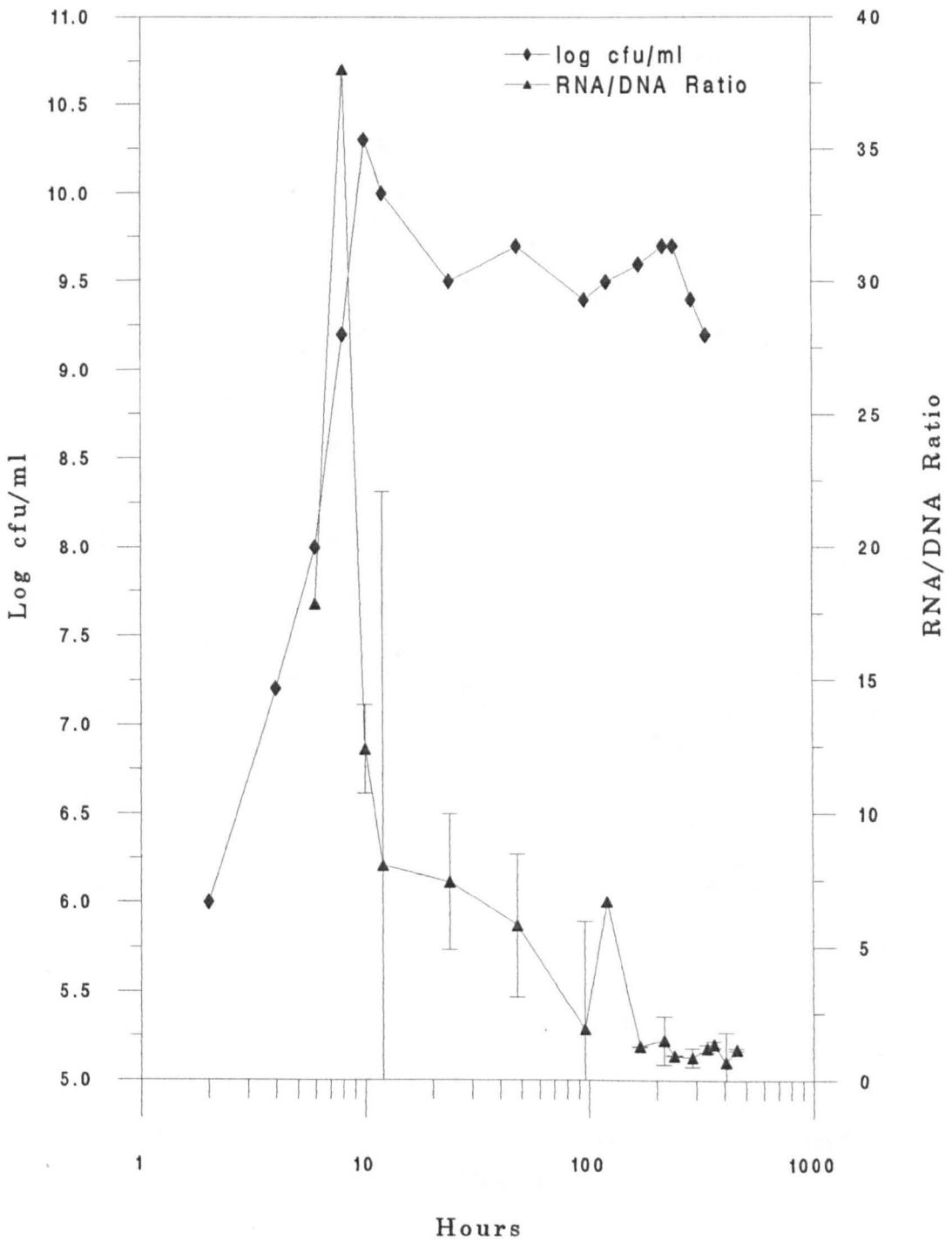
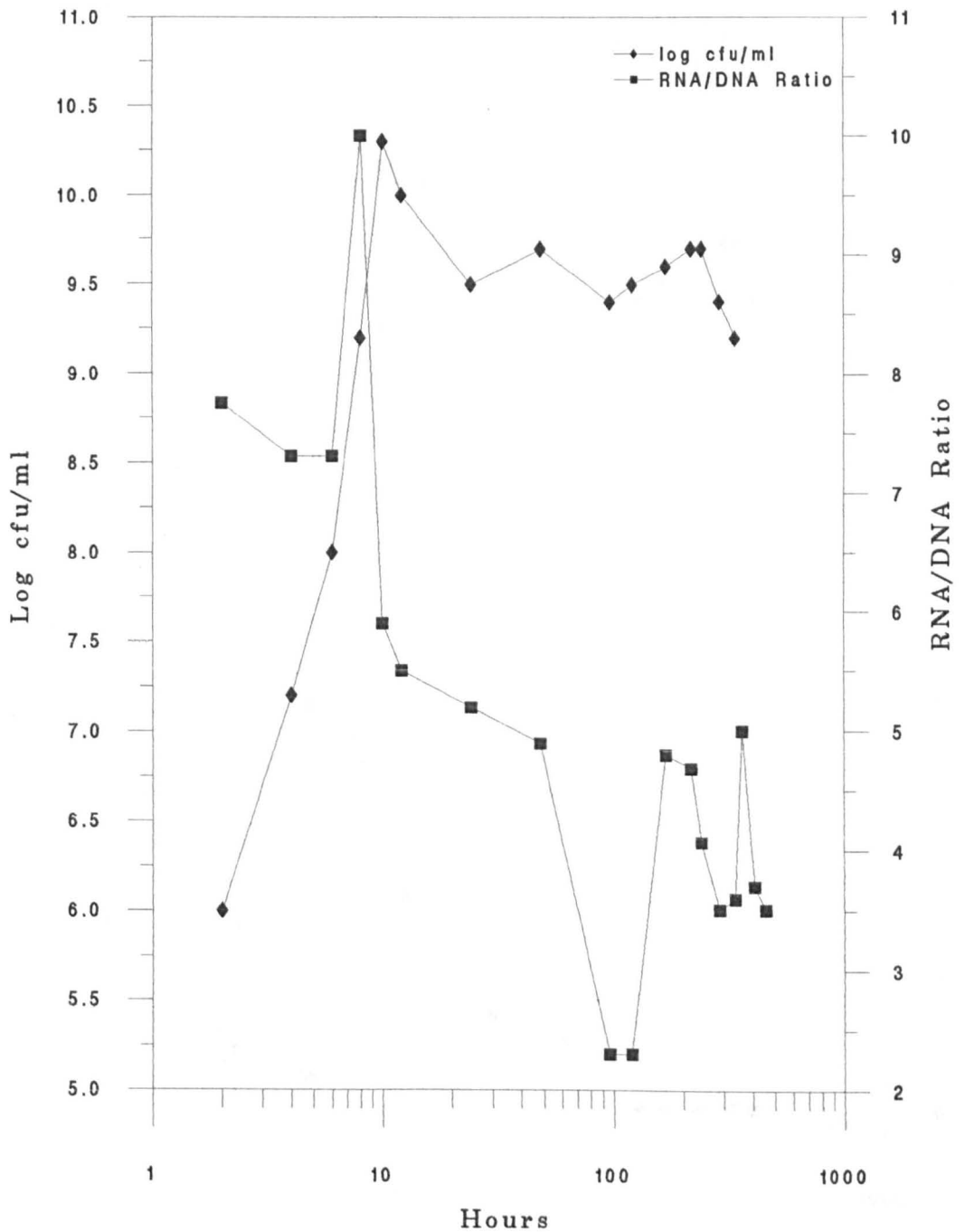
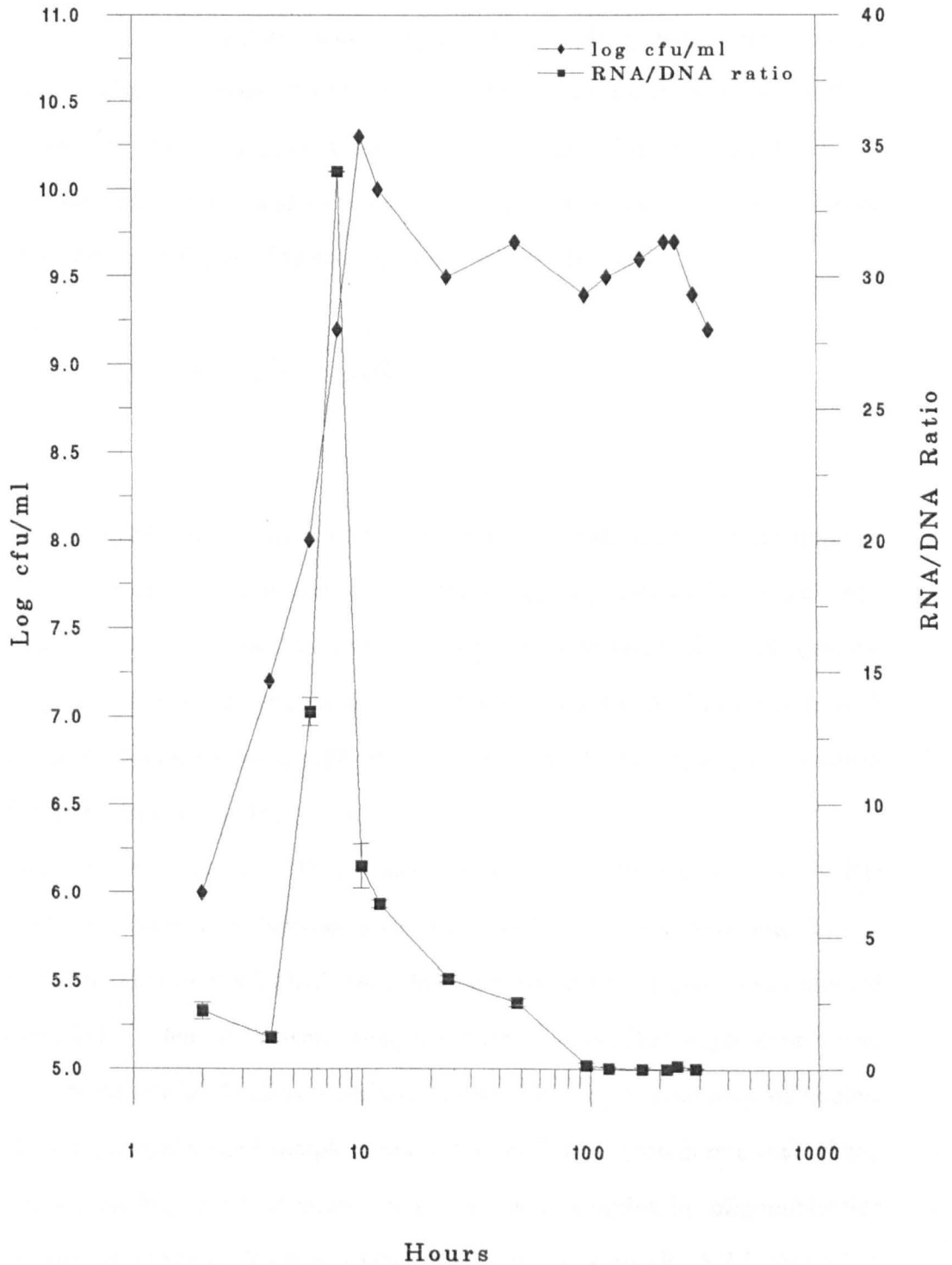


Figure 5.6.2.4. Nucleic acid ratios measured by fluorometry of nucleic acid extracts over batch growth of L.monocytogenes (cfu/ml).



N.B. Values are result of triplicate means. Standard deviations <+/-15% of mean.

Figure 5.6.2.5. Growth of L. monocytogenes in pH controlled batch culture and RNA/DNA ratios monitored by oligonucleotide probing.



5.7.1. Nucleic acid ratios as a function of growth rate.

When minimal medium was supplemented with growth rate limiting concentrations of carbon source (0.174 - 43.5 mmoles glucose) and inoculated with *L. monocytogenes* the following growth curves were obtained, (Fig. 5.7.1.). At the two sampling points indicated (11 and 13 h. incubation) colony counts (Table 5.7.1.) were used to determine growth rate. The equation used was as follows:

$$\mu = \frac{(\log N - \log N_0) \times 2.303}{t - t_0}$$

where μ = growth rate hours^{-1}; t_0 = initial sampling time (hours); t = time at sampling point t + 1 (hours); log N_0 = log cfu.ml^{-1} at initial sampling point; log N = log cfu.ml^{-1} at sampling point t + 1. The relationship between growth rate and mmol. glucose added is shown in Fig. 5.7.2. Oligonucleotide probing of nucleic acid extracts showed an increase in RNA concentration with respect to growth rate, but DNA concentration remained largely unchanged (Fig. 5.7.3.).

Analysis of RNA and DNA concentration by flurometry of whole cells showed a positive correlation between growth rate and RNA/DNA ratio (Fig. 5.7.4.). However, differences in RNA/DNA ratio between successive growth rates studied were insignificant when data were analysed with a two-tailed significance test. Representative nucleic acid ratios were difficult to obtain by fluorometry of nucleic acid extracts, particularly from samples removed at the lowest growth rate studied and are not shown on Fig. 5.7.4. Analysis of nucleic acid samples by oligonucleotide probing (standard curves of RNA and DNA are shown as appendix 5.7.1. and 5.7.2. respectivley) indicated a positive relationship between RNA/DNA ratio and growth

Table 5.7.1. Growth rates of *L. monocytogenes* in Trivett and Meyer medium containing various concentrations of glucose.

Glucose mmol.l^{-1}	log N_0	log N	μ (hours^{-1})
0	7.12	7.15	0.069
0.151	7.30	7.35	0.115
0.757	7.65	7.73	0.184
1.51	7.70	7.90	0.461
2.5	7.75	8.05	0.691
7.5	7.75	8.05	0.691
22.7	7.70	8.10	0.921
37.8	7.50	7.95	1.036

Figure 5.7.1. Growth of L. monocytogenes (O.D. 660nm) in minimal media supplemented with different concentrations of glucose.

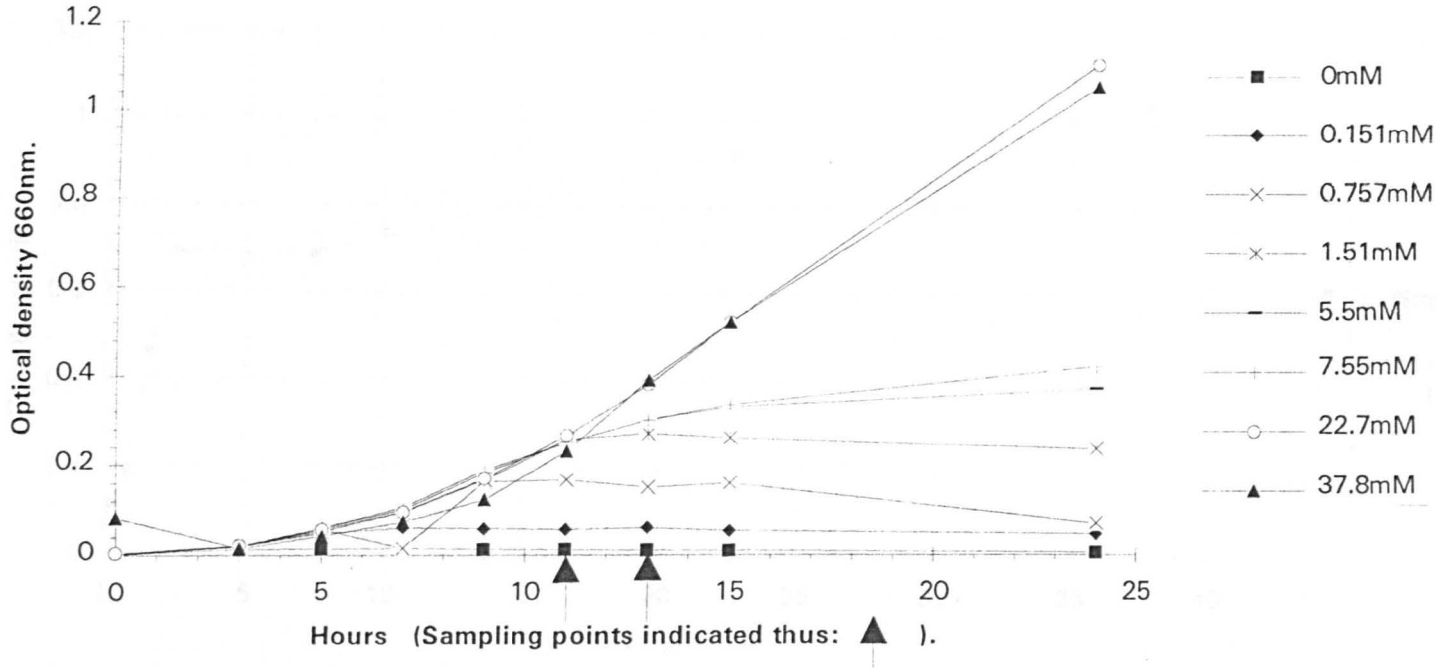
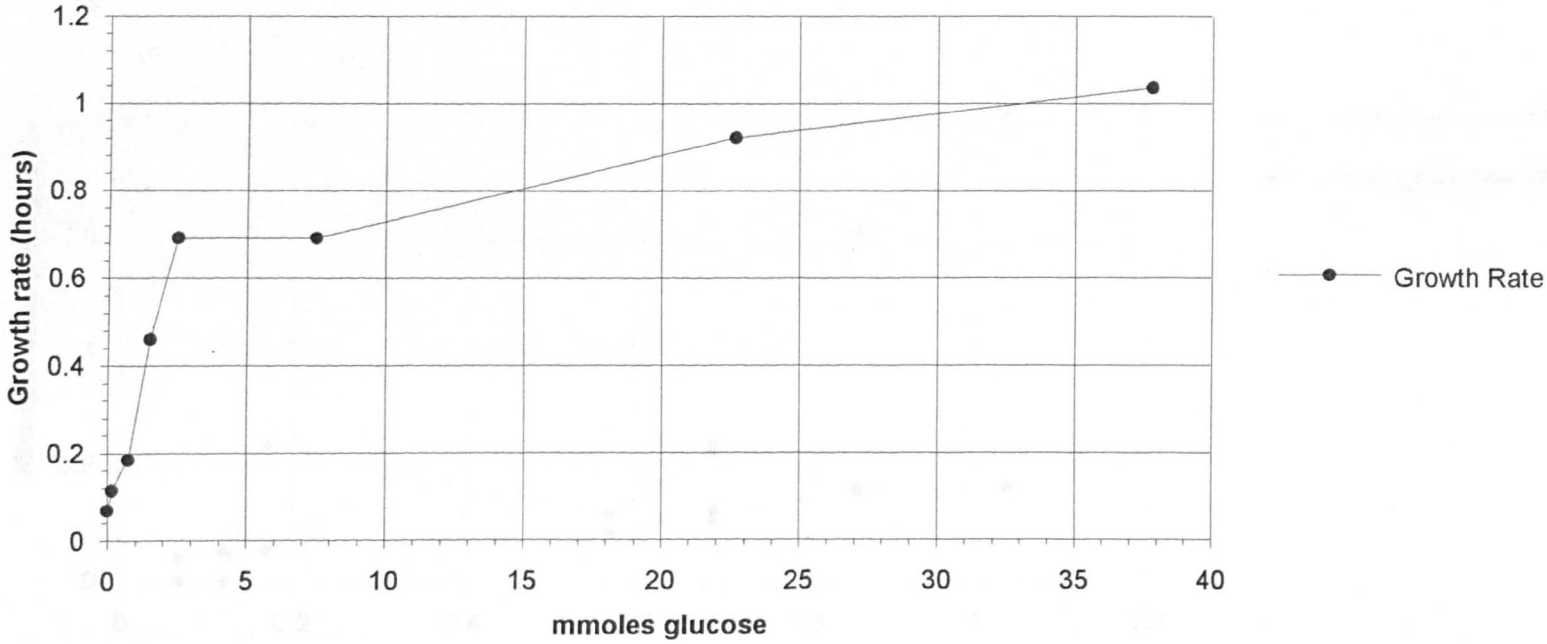
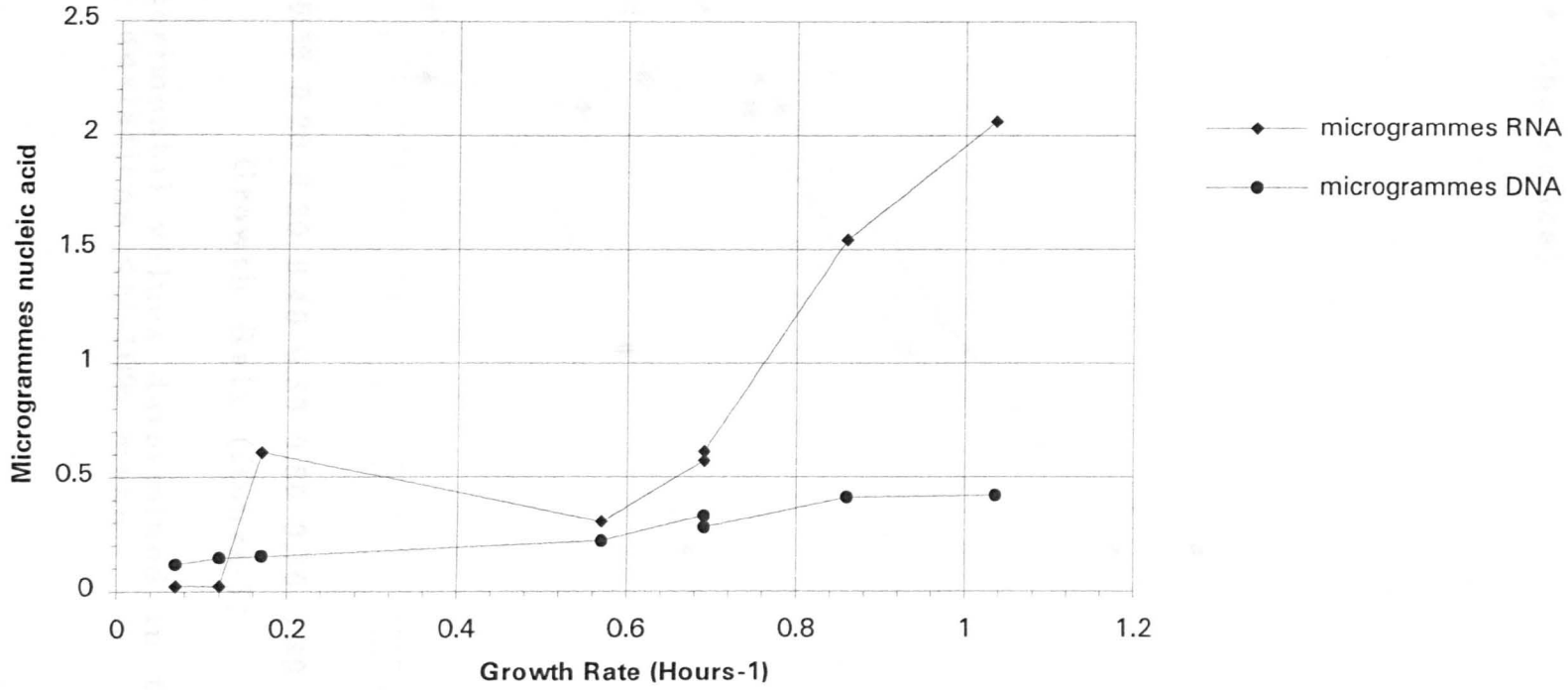


Fig. 5.7.2. Growth rate of L. monocytogenes against mmoles of glucose in minimal media



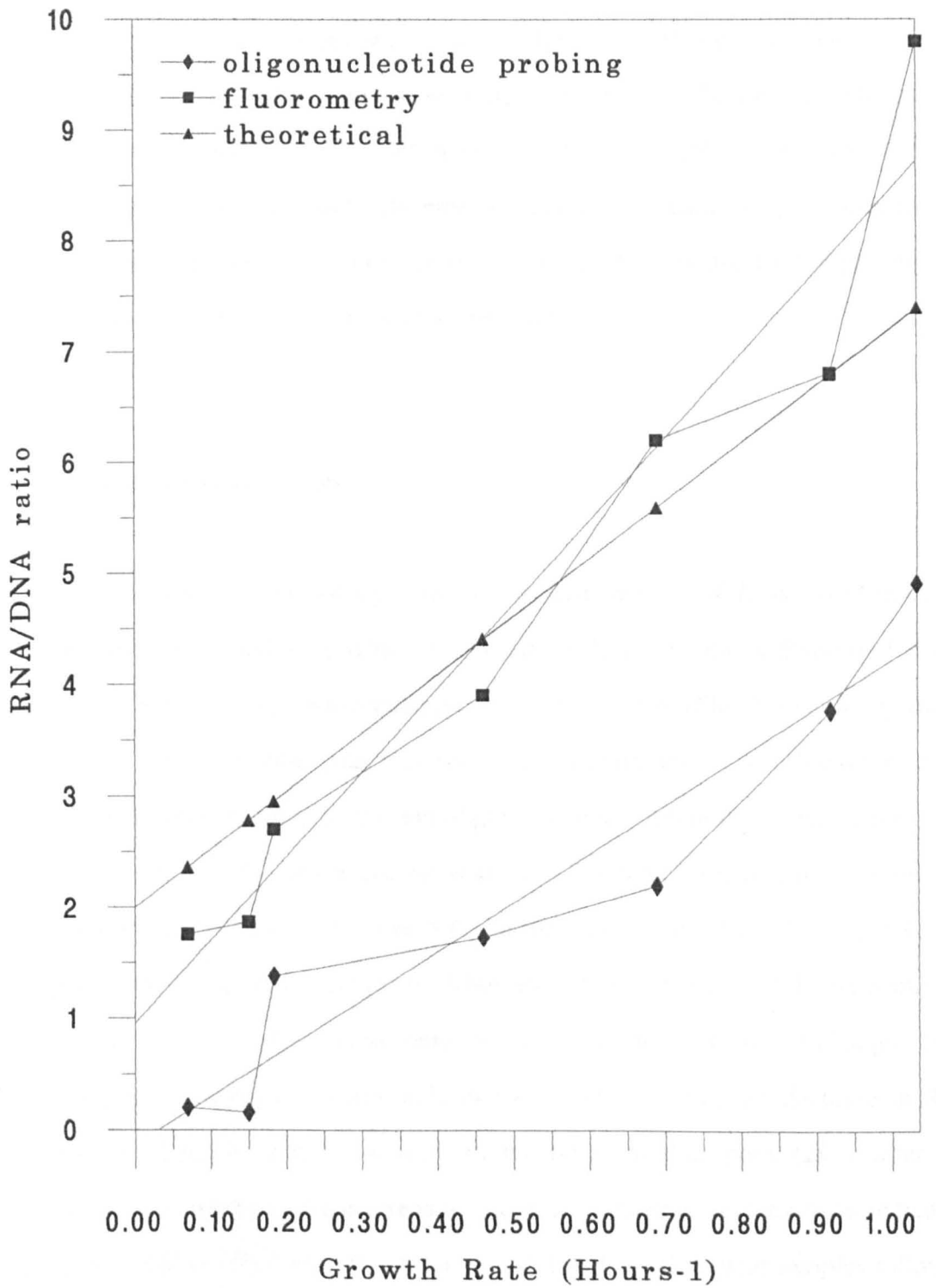
N.B. Points determined in triplicate series. Standard deviations <+/- 10% mean.

Figure 5.7.3. Growth Rate of L. monocytogenes against microgrammes of nucleic acid per 10exp.9 cells measured by oligonucleotide probing.



N.B. Points represent trilplicate means. Standard deviations <10% +/- mean.

Figure 5.7.4. Nucleic acid ratios from L. monocytogenes at various growth rates determined by fluorometry and oligonucleotide probing.



N.B. Experimental values determined in triplicate. standard deviations <+/-10% mean.

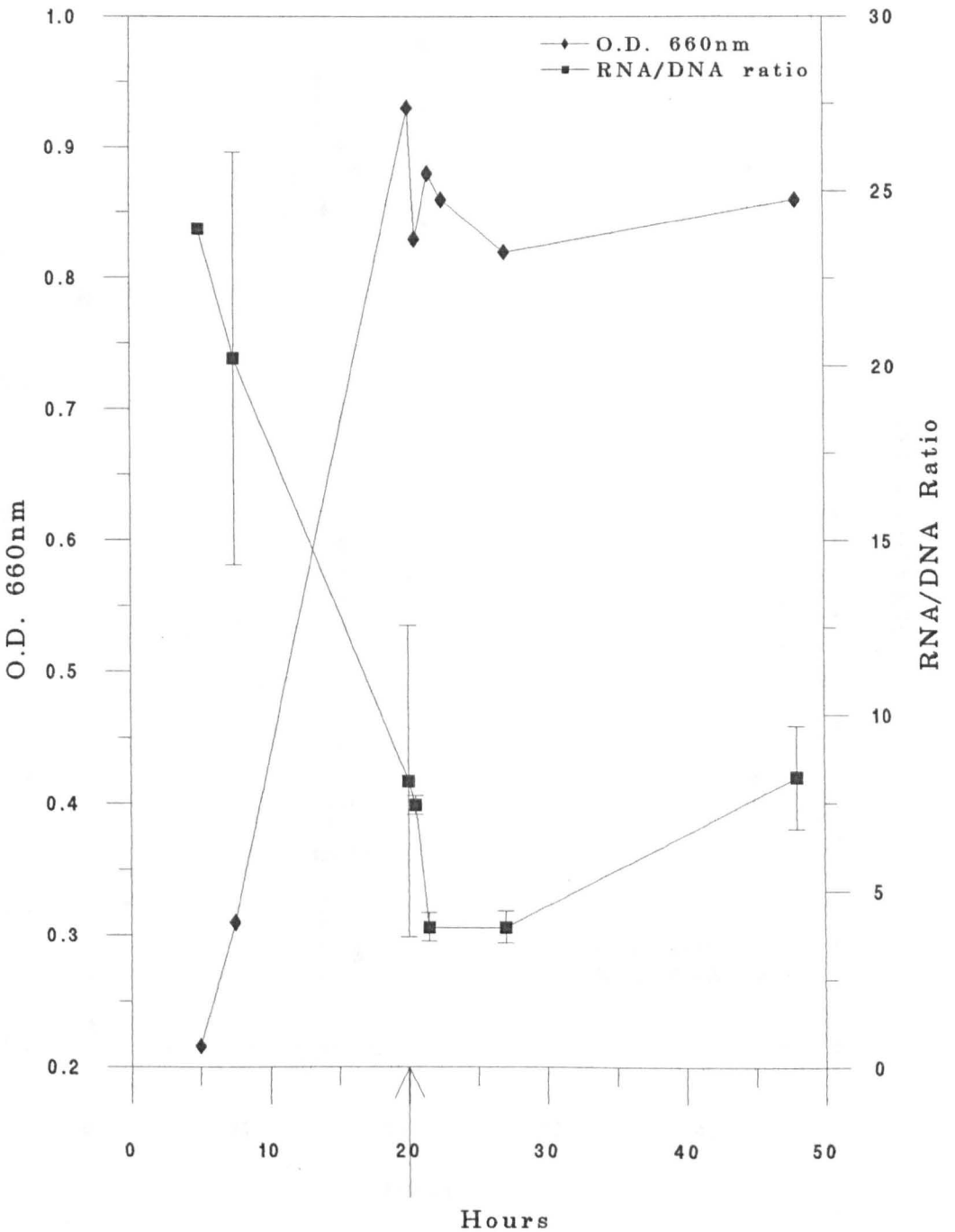
rate (Fig. 5.7.4.), although individual differences in nucleic acid ratios between successive growth rates were statistically insignificant. Regression analysis by the least square method and equations describing the relationship between growth rate and RNA/DNA ratio are as follows: RNA/DNA = -0.145 + 4.34(μ) and RNA/DNA = 0.96 + 7.50(μ) for oligonuclotide probing and fluorometric analysis respectively. Correlation coefficient (r^2) values are 0.93 and 0.95 for oligonucleotide probing and fluorometeric analysis of whole cells, respectivley.

5.8. Acid shock experiments.

Exposure of a stationary phase batch fermentation of *L. monocytogenes* in Trivett and Meyer defined medium to pH 3.0 for 30min by the addition of 1M HCl caused a small, but significant decrease in optical density (Fig. 5.8.1). A significant change in colony forming units was not observed (data not shown). Measurement of RNA/DNA ratios by fluorometry and oligonucleotide probing of nucleic acid extracts showed significant changes in nucleic acid ratios 5 h. after fermentor inoculation until the addtion of HCl (Figs. 5.8.1. and 5.8.2. respectively; appendix 5.8.1. and 5.8.2. for oligonucleotide calibration curves for RNA and DNA, respectivley). Upon addtion of HCl, changes in nucleic acid ratio varied dependant on the technique used. Fluorometric analysis of nucleic acid extracts show a significant decrease in RNA content, followed by a slow recovery by the final sampling point (24 h. after acid addition). Quantitative oligonucleotide probing of nucleic acid extracts indicate a significant rise in RNA content after acid addition, but subsequent samples follow no particular pattern.

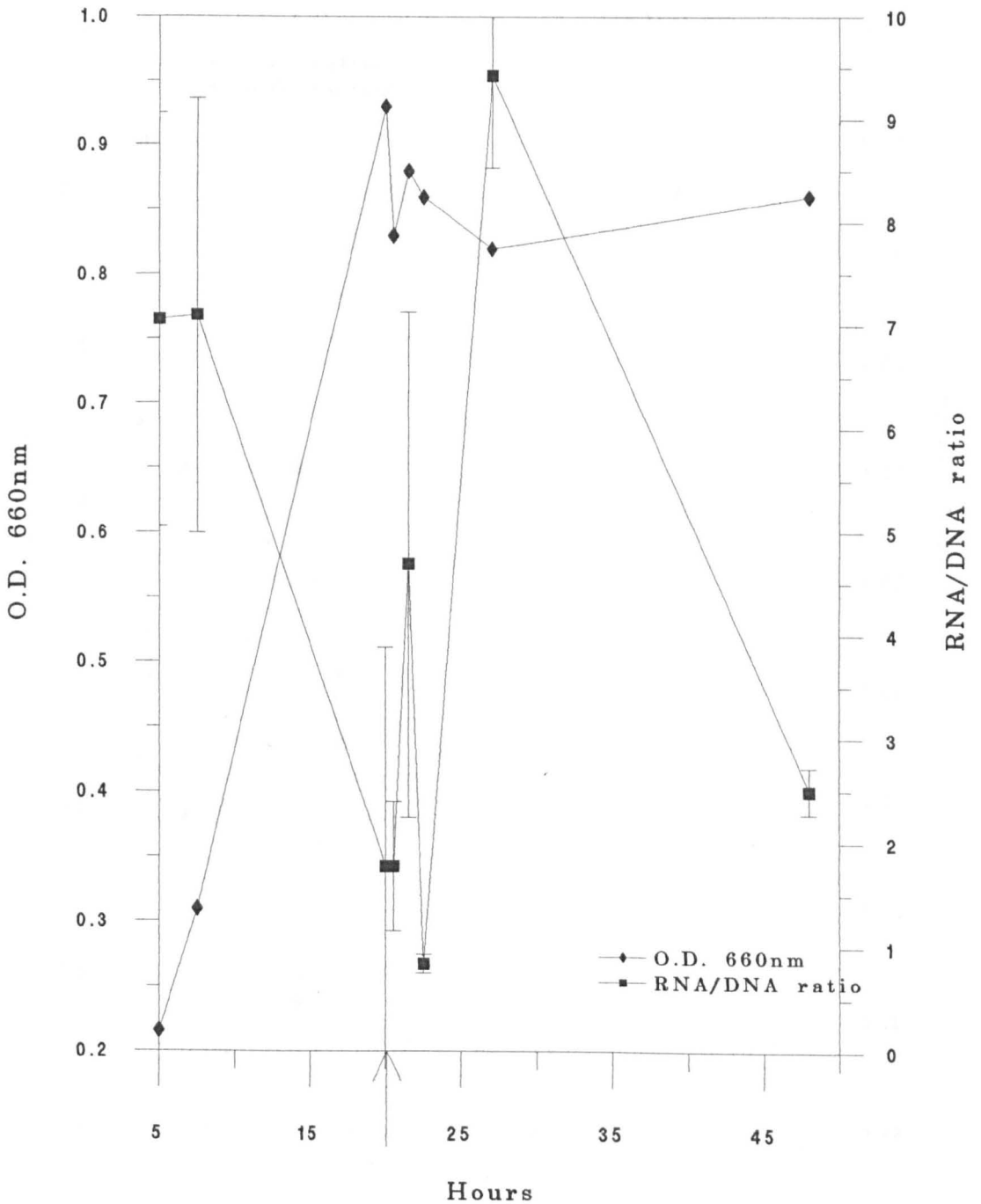
When *L. monocytogenes* was cultured in Trivett and Meyer minimal media to mid-exponential phase and culture pH lowered to pH 3.0 with HCl for 30min a significant change in the number of colony forming units was observed (Fig. 5.8.3.).

Figure 5.8.1. Effect of acid addition on growth of L. monocytogenes and RNA/DNA ratios measured by fluorometry.



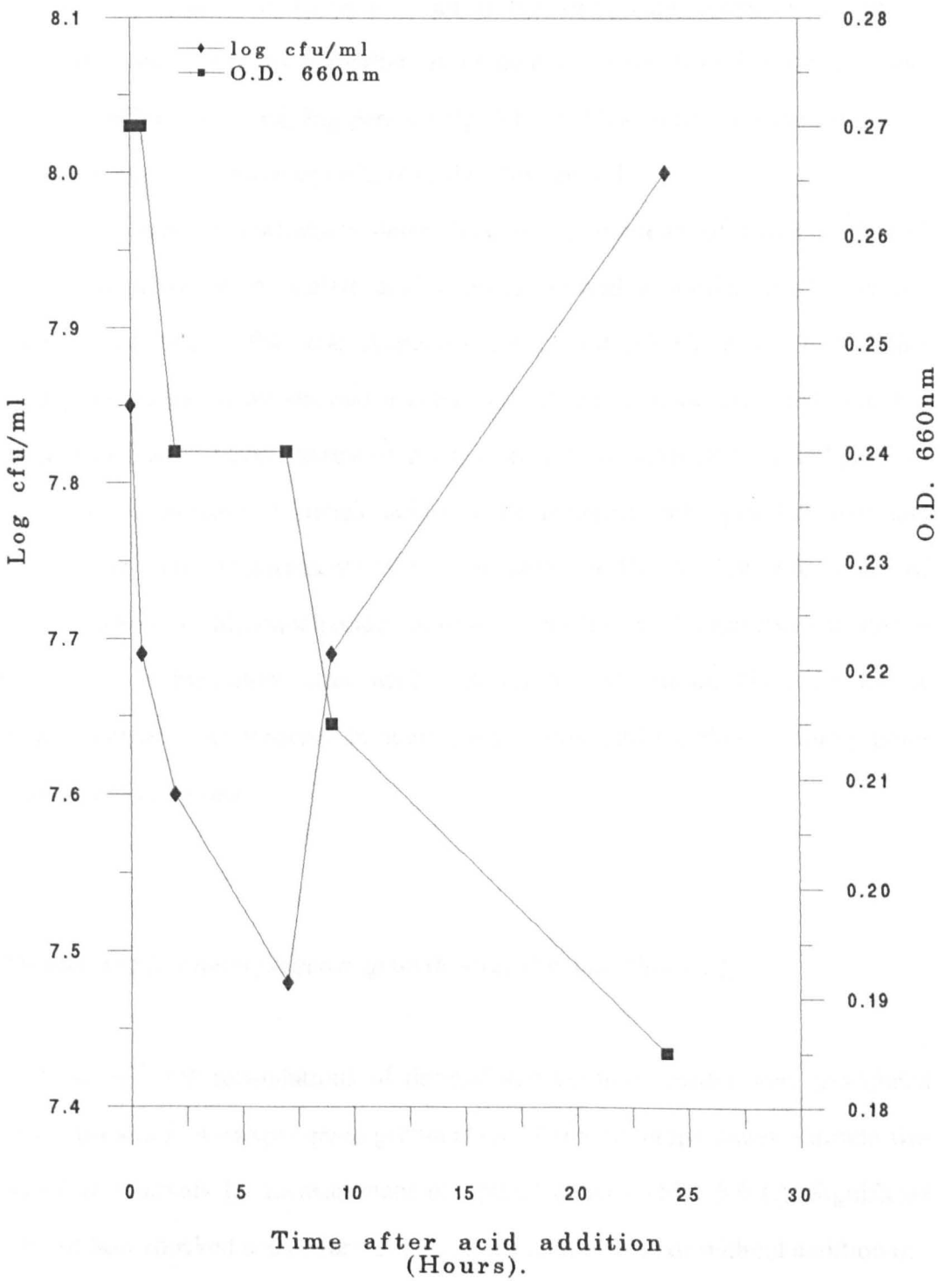
N.B. Point of acid addition indicated thus: ↑

Figure 5.8.2. L. monocytogenes growth and RNA/DNA ratios measured by oligonucleotide probing after acid exposure.



N.B. Point of acid addition indicated thus: ↑

Figure 5.8.3. L. monocytogenes growth after acid addition during mid-exponential growth.



N.B. Traces are triplicate means.

Colony forming units were slightly lowered after the addition of HCl, but an increase is observed 2 h. after acid addition. Gradual but significant decreases in colony forming units were observed thereafter. A gradual decrease in optical density was observed throughout the sampling period (Fig. 5.8.3.). This contradicts data from the determination of colony forming units over the same period.

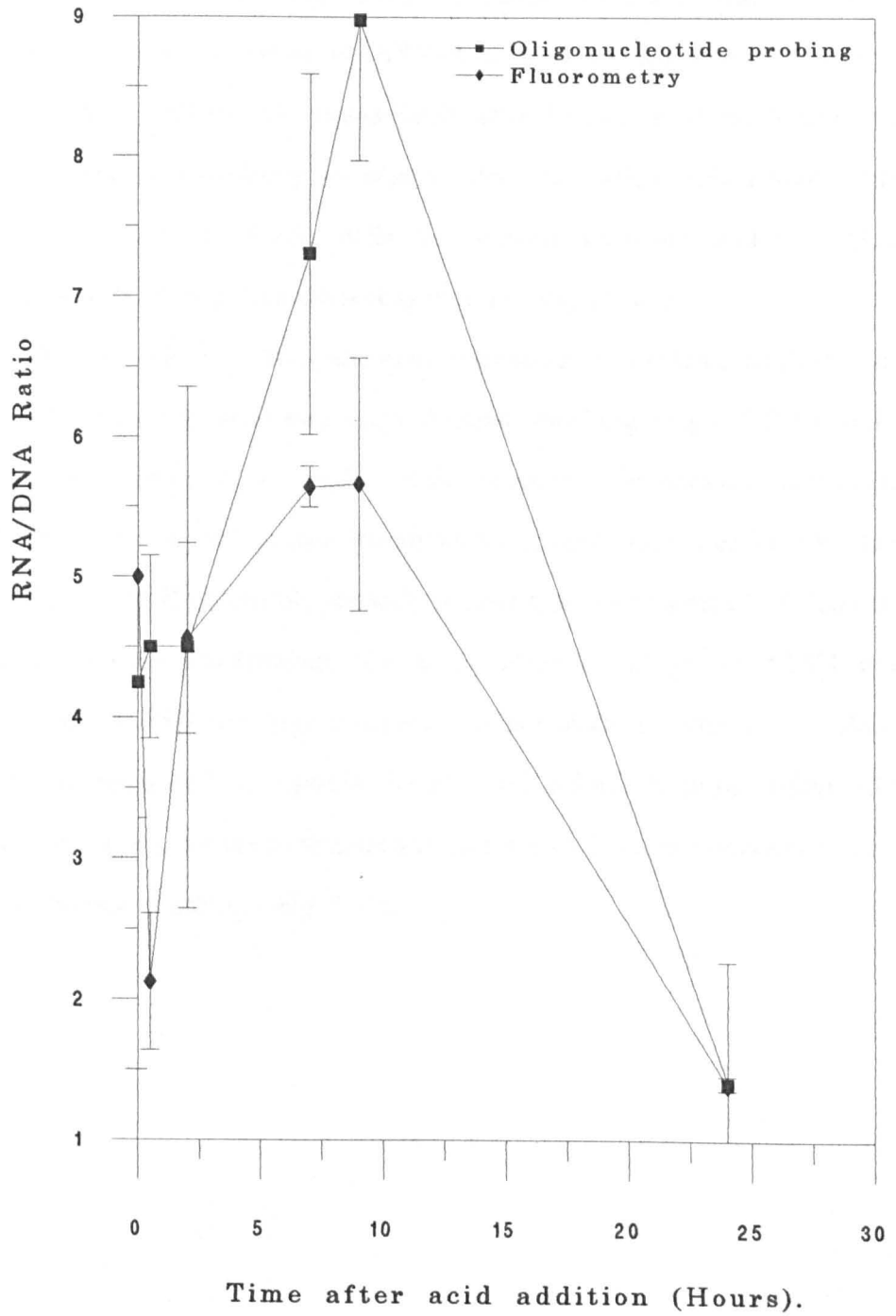
Nucleic acid concentrations determined by fluorometry of whole cells and oligonucleotide probing of nucleic acid extracts showed a similar trend over the sampling period (Fig. 5.8.4. and Appendix 5.8.2. and 5.8.3.). RNA/DNA ratios determined fluorometrically showed a substantial decrease upon HCl addition, but ratios recovered and indeed, increased when compared to samples analysed prior to acid addition. A decrease in nucleic acid ratios concurrent with optical density and colony forming unit measurements were seen between 10 and 24h. Measurent of RNA/DNA ratios by oligonucleotide probing of nucleic acid extracts indicated a slight increase immediatley after acid addition to the culture (Fig. 5.8.4.). A significant decrease was observed in nucleic acid ratios until the final sampling point (24 h. after acid addition).

5.9. Monitoring *L. monocytogenes* growth after thermal shocking.

Four different formulations of defined and complex media were inoculated with heat shocked *L .monocytogenes* grown at two different temperatures. Growth was monitored at intervals by measurement of optical density (Fig. 5.9.1.). Significant recovery of heat shocked cells ocurred in complex media, with or without addition of 1% (v/v) pyruvate. *L. monocytogenes* inocula grown at 40°C recovered (to O.D. 660nm = 0.72 after 11 h.) significantly quicker than those grown at 25°C (O.D. = 1.04 after 24 h.).

Unfortunately, recovery in defined media preparations and complex media inoculated with cells grown at 25°C was insufficient to permit extraction of nucleic

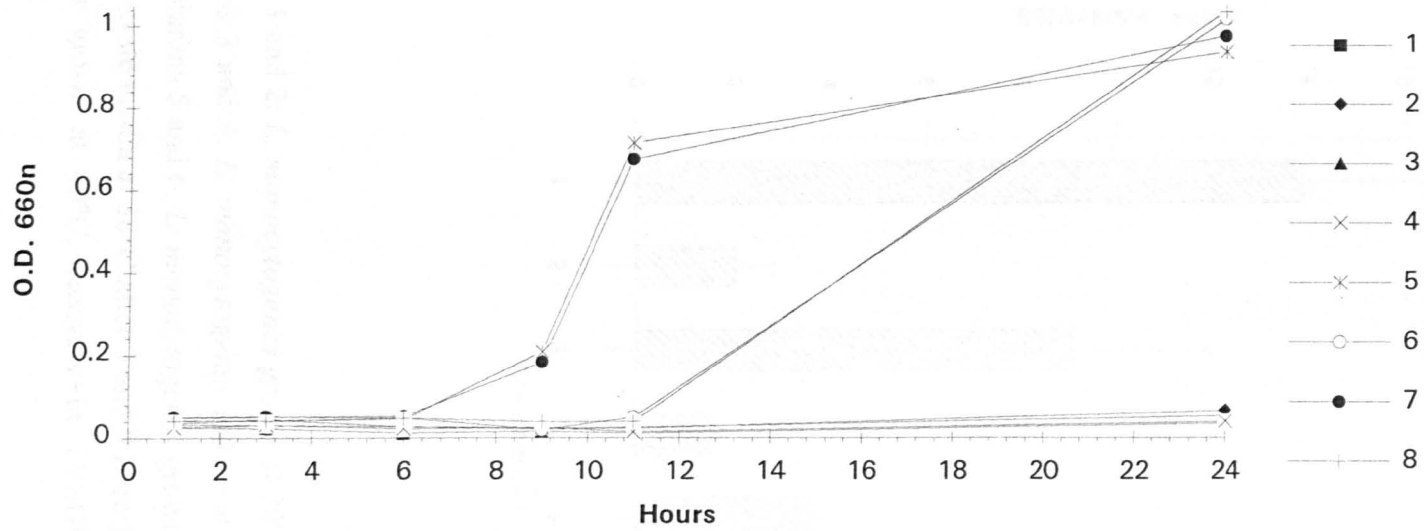
Figure 5.8.4. RNA/DNA ratios determined by oligonucleotide probing and fluorometry after acid shocking during mid-exponential growth.



acids from 1ml culture over the course of this study. Therefore nucleic acids were successfully sampled only from complex media inoculated with *L. monocytogenes* cells grown at 40°C. However, RNA/DNA ratios were measured for both cells grown at 25° and 40°C, prior to, and immediately after thermal shocking. Nucleic acid ratios were obtained by flurometry of whole cells and nucleic acid extracts taken from recovery in TSYGB media with or without pyruvate addition. Quantitative oligonucleotide probing was unsuccessful in this experiment.

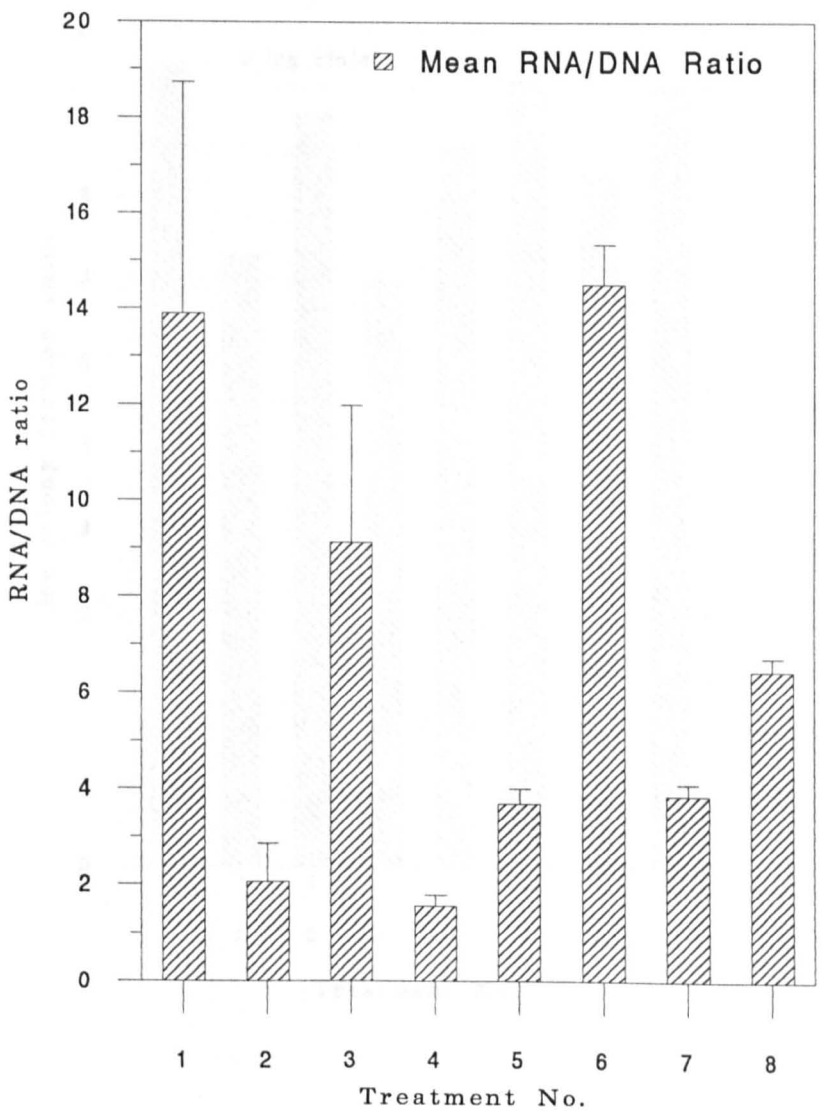
Both nucleic acid ratios and colony forming units calculated from cell samples exhibited significant decreases upon thermal shocking (Figs. 5.9.2.a and 5.9.2.b.. respectively). Recovery in complex media resulted in increased in cell numbers and RNA/DNA ratio in both media formulations studied. Increases in RNA/DNA ratio detected in TSYGBP were significantly greater than those seen in TSYGB alone over the period studied. The recovery of *L. monocytogenes* cell grown at 25°C in complex media is noteworthy. Athough recovery was not observed after 11h. in these media, growth (as measured by optical density and colony forming units) after 24 h. incubation was equivalent to that seen in cultures of *L. monocytogenes* grown at 40°C prior to thermal shocking (Fig. 5.9.2).

Fig. 5.9.1.Recovery of L. monocytogenes in various media after thermal injury.



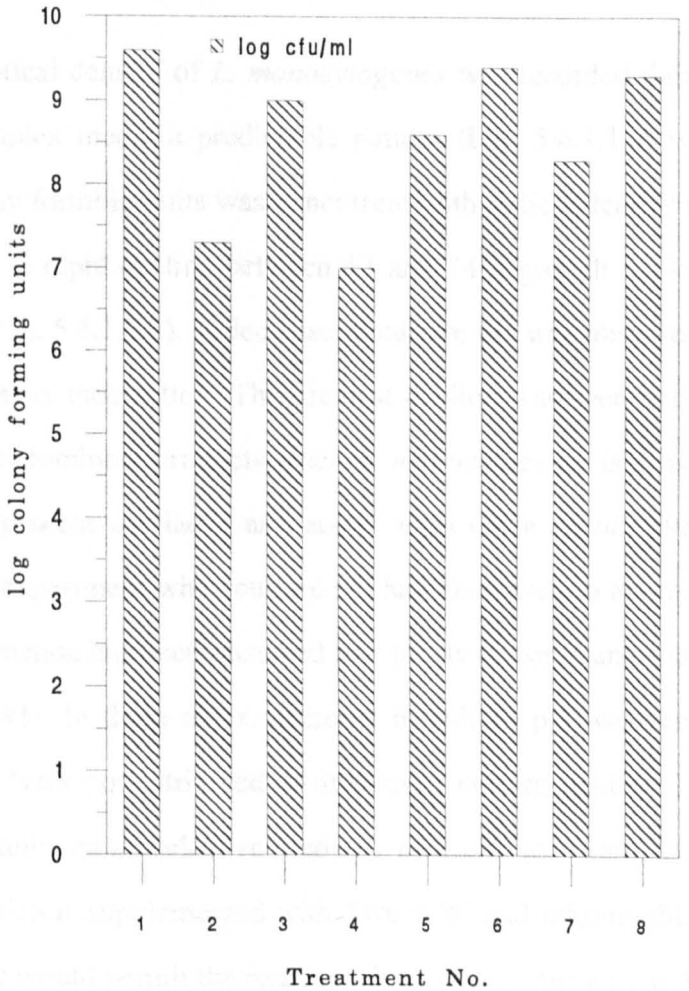
N.B. Optical density determined in triplicate series. Standard deviation < +/- 5% of mean. Media formulation: 1, Trivett and Meyer medium + 1% (v/v) pyruvate (TMP) cells cultured at 40°C prior to heat-shock; 2, TMP, cells cultured at 20°C prior to heat-shock; 3, Trivett and Meyer medium (TM), culture at 20°C; 4, TM medium cultured at 40°C; 5, TSYGB + 1% (v/v) pyruvate (TSYGBP) cells cultured at 40°C; 6, TSYGB cultured at 20°C; 7, TSYGB cultured at 40°C; 8, TSYGBP cultured at 20°C.

Figure 5.9.2.a. RNA/DNA ratios of L. monocytogenes before and after heat shock in different recovery media.



N.B. Columns 1 and 2: *L. monocytogenes* grown at 20°C before (1) and after (2) heat shock. Columns 3 and 4: *L. monocytogenes* grown at 40°C before (3) and after (4) heat shock. Columns 5 and 6: *L. monocytogenes* grown at 40°C, recovery in TYSGB + 1% (v/v) pyruvate media at 30°C after 7 and 9 h. respectively. Columns 7 and 8: *L. monocytogenes* grown at 40°C, recovery in TYSGB at 30°C after 7 and 9 h. respectively.

Figure 5.9.2.b. Number of L. monocytogenes measured before and after heat-shock in a variety of growth media.



N.B. Columns 1 and 2: *L. monocytogenes* grown at 20°C before (1) and after (2) heat shock. Columns 3 and 4: *L. monocytogenes* grown at 40°C before (3) and after (4) heat shock. Columns 5 and 6: *L. monocytogenes* grown at 40°C, recovery in TYSGB + 1% (v/v) pyruvate media at 30°C after 7 and 9 h. respectively. Columns 7 and 8: *L. monocytogenes* grown at 40°C, recovery in TYSGB at 30°C after 7 and 9 h. respectively. Determinations performed in triplicate. S.D. < +/- 10% of mean.

5.10. DISCUSSION.

5.10.1. RNA/DNA ratios during *L. monocytogenes* batch culture.

When optical density of *L. monocytogenes* was recorded during growth in an un-buffered complex media a predictable pattern (Fig. 5.6.1.1.a) was obtained. An increase in colony forming units was concurrent with optical density increase, but this was followed by a rapid decline between 13 and 24 h growth whilst optical density remained high (Fig. 5.6.1.1.b). A decrease in culture pH was observed from pH 6.5 to pH 4.1 at 72 h after inoculation. The greatest decline was seen between inoculation and 13 h. The predominant products when *L. monocytogenes* is grown aerobically in the presence of glucose are lactic and acetic acids (Pine *et. al.*, 1989). Growth was inhibited in this experiment when culture pH had decreased to approximately pH 4.3. A similar phenomenon has been observed previously (George and Lund, 1992 and ter-Steeg *et. al.*, 1991). In these cases, decrease in culture pH was accompanied by an increase in cell lysis not attributed to increased culture acidity. Determination of colony forming units on an selective recovery medium, for example modified Vogel-Johnson agar which is supplemented with Tween-80 and lithium chloride (Smith and Buchanan, 1990) would permit the relationship between time and optical density to be further defined under these conditions. This selective medium has been used to recover injured *L. monocytogenes* cells and would permit determination of injured, and total cell populations.

Measurement of growth by optical density and colony forming units highlights the disadvantage of the former technique when used in isolation. Optical density has been shown to have a low sensitivity ($<10^5$cfu.ml^{-1}, this study), effects of cell size, aggregation or physiological state are not discriminated.

Standard curves of nucleic acid concentration against pixel sum above background show second order polynomial relationships for both RNA and DNA (Fig. 5.6.1.3. and 5.6.1.4.). These permit valid calculation of experimental nucleic

acid concentrations in the range of 1 - 24μg RNA and 0.01 - 24μg DNA. Whilst correlation coefficients are good for both nucleic acids, the standard curve of RNA is founded on only six concentrations. RNA concentration and pixel intensity at both extremes of RNA concentration cannot be accurately defined on the basis of this relationship, and this affects confidence in experimental values of RNA concentration. Future experiments employed 8 - 12 nucleic acid samples of known concentration.

Nucleic acid ratios measured by fluorometry of nucleic acid extracts and oligonucleotide probing show similar relationships with respect to *L. monocytogenes* growth over time (Fig. 5.6.1.5. and 5.6.1.6. respectivley). In both examples RNA/DNA ratio increases to maxima at the end of exponential growth (12 h.). Nucleic acid ratios declined rapidly in subsequent samples. Nucleic acid ratios determined by these techniques show similar trends over time. Total RNA present is recorded by fluorometry and are consequently an overestimate of RNA/DNA ratio would be expected. However, 85% of RNA contained in rapidly growing cells is assumed to be rRNA (Bremer and Dennis, 1987). Therefore, estimation of RNA content by fluorometry at rapid growth rates predominantly reflects rRNA content.

Growth of *L. monocytogenes* in batch culture continues over a longer period when culture pH is controlled (Fig. 5.6.2.2.). Measurement of optical density and colony forming units showed similar dynamics over the growth phase; maximum growth was determined at 10 and 12 h. by optical density and colony forming units respectively. Nucleic acid ratios determined by fluorometry of whole cells, nucleic acid extracts and oligonucleotide probing showed similar patterns during batch culture (Figs. 5.6.2.3., 5.6.2.4. and 5.6.2.5. respectively). RNA/DNA ratios increased rapidly from inoculation to a maximum after 8 h. growth. Subsequent ratios fell rapidly and further decreases were gradual despite colony forming units remaining high (10^9 $cfu.ml^{-1}$). Although trends in nucleic acid ratios are similar for the techniques used, those measured by fluorometry of nucleic acid extracts were considerably lower than those measured by fluorometry of whole cells or oligonucleotide probing. Possible reasons are discussed in section 5.10.5.

In batch culture, the expected growth rate (μ) will tend towards μmax. Maximum specific growth rate is dependent upon affinity to a given substrate (K_S; Monod, 1949). In complex medium a number of substrates may contribute to growth rate limitation, and indeed may be utilised sequentially. However, more abrupt changes between growth phases are observed when a defined medium with a single growth limiting nutrient is used (Neidhardt *et. al.*, 1990). Future studies on the effects of starvation on *L. monocytogenes* should employ such a medium to permit more accurate definition of growth phases.

The relationship between RNA/DNA ratio and growth rate of bacteria has been extensively studied using a variety of methods (Gausing, 1977; Kerkhof and Ward, 1993; Kjellgaard and Kurland, 1963; Moyer *et. al.*, 1990; Rosett *et. al.*, 1966), but relatively little attention has been given to nucleic acid ratios observed in batch culture conditions. Excepting cells in exponential phase of growth, a sample removed at any other phase of batch culture will contain a heterogeneous population of cells; i.e.: unbalanced growth. Therefore, a sample taken in early death phase will contain cells which may be in active stages of growth and division, are inert, dead, or non-recoverable. The techniques employed in these studies are non-discriminatory in this respect. Therefore, RNA/DNA ratios observed are a crude average of the cell population status at a given instance. However, RNA/DNA ratios observed here were at maximal at high growth rate. Samples removed at 2 and 4 h. from batch culture analysed by oligonucleotide probing showed similar nucleic acid ratios to those analysed from 24 and 48 h. (Fig. 5.6.2.5.). This is a clear demonstration that measurement of RNA/DNA ratio alone does not discriminate a lag phase from a stationary phase culture. Nucleic acid ratios may decrease rapidly prior to maximum cell numbers because rapidly growing cells contain a number of chromosomal DNA replication forks (Bremer and Dennis, 1987). The cell is committed to division but limitation of nutrients means that cell size and translation of cell macromolecules are reduced. Therefore, although cell numbers increase, the relative amount of RNA

decreases. Consequently, cell numbers are not accurately represented by nucleic acid ratios in this situation.

Dynamics of RNA content during batch growth similar to those determined here have been successfully monitored by measurement of radioactive labelled uridine content in batch growth of *E. coli* (Davis *et. al.*, 1986). Loss of ribosome function, 30S subunit degradation and subsequent cell death were observed during *E. coli* cell starvation (Kaplan and Apirion, 1974). Although RNA/DNA ratios were seen to slowly decrease from 10 h. until the end of the experiment, cell numbers remained high (log cfu/ml = 9.1). Previous studies have shown that ribosomes serve as both energy and nutrient sources for starved *E. coli* cells (Kaplan and Apirion, 1975). Numbers of *L. monocytogenes* cells remain high after 24 h. culture possibly due to scavenging material from dead cells and utilisation of ribosomal material. Minimum nucleic acid ratios observed in cultures over this experiment were 3.5, 0.69 and 0.25 obtained by fluorometry of nucleic acid extracts, whole cell fluorometry and oligonucleotide probing respectively. Ratios were determined from 336 to 456 h. and represent minimum RNA/DNA ratios observed where *L. monocytogenes* survival is extensive. Assuming the equation to calculate ribosome numbers (Kerkhof and Ward, 1993) where:

$$\text{ribosome number} = \frac{\text{RNA/DNA ratio} \times (C)}{R}$$

[C = chromosome length in bp. (3.15×10^6); R = length of 23S+16S+5S rRNA in bp. (4566)] ribosome numbers are between 200 and 900 per cell between 336 and 456 h.. The extent of this survival is indeed remarkable, and demonstrates that toxic metabolites were not produced or deposited in sufficient quantities to cause cell death.

5.10.2. RNA/DNA ratio with respect to growth rate.

Growth of *L. monocytogenes* in minimal medium containing various concentrations of a carbon source was monitored by measuring optical density (Fig.

5.7.1.) This indicates that quantity of carbon source limits the growth of *L. monocytogenes*. However, when optical density indicates exponential growth phase cultures appear to grow with similar gradients which are representative of growth rate (Neidhardt *et. al.*, 1990). Samples were removed at 11 and 13 h. and viable counts assessed. This data was used to calculate growth rates presented in (Table 5.7.1.). The relationship between growth rate and concentration of glucose (Fig. 5.7.2.) was similar to that found by Monod (1948). A rapid initial increase in growth rate is seen with respect to carbon source concentration. Subsequent increases are gradual, possibly indicative of growth rate, μ tending to the maximum specific growth rate (μ max) for glucose. However, despite growth rate determination, macromolecular composition is unlikely to be representative. When sampled, *L. monocytogenes* was not growing at an exponential rate, i.e. growth was not balanced. Consequently, samples will have comprised cells growing at different rates and of different macromolecular composition. Growth rate may be manipulated with increased rigour by control of a growth rate limiting substrate in continuous culture of the organism in balanced growth. Continuous culture of *L. monocytogenes* in Trivett and Meyer (1971) medium with glucose as the rate limiting substrate was attempted over a lengthy period. This was achieved with limited success but considerable problems were encountered in maintaining sterility. This was a particular problem because some media components could not be autoclaved, and had to be added as a filter-sterilsed solution to the minimal salts solution. Additional problems were encountered when changing media reservoirs.

Alternativley, growth rate may be manipulated by use of different carbon sources (Gausing, 1977; Kjelgaard and Kurland, 1962). Whilst this is convenient, it should be noted that different carbon sources may be assimilated by different mechanisms. This has been proposed for *E. coli* growing in media containing either glucose, glycerol, lactose, maltose or melebiose (Dillis *et. al.*, 1980). It was proposed that dependent on concentration, glucose is metabolised by a combination of pathways of differing efficiency (Shehata and Marr, 1970), which in turn, affect

growth rate. Due to the differing energetic requirements of carbon source uptake, maintenance energy requirements for growth on each substrate will be altered (Pirt, 1965). Therefore, to limit the number of variables encountered it is advantageous to alter growth rate limitation of one essential nutrient. The concentration of a given nutrient at which growth rate is limited is often in the order of micromoles (Neidhardt, *et. al.*, 1990), and consequently cell mass produced are insufficient for many analytical methods. In conclusion, growth rate should ideally be manipulated by continuous culture with one growth rate limiting substrate. However, when apparent growth rate and nucleic acid concentrations were assayed by oligonucleotide probing of nucleic extracts an interesting relationship was observed (Fig. 5.7.3.). Concentration of DNA per 10^9 cells was almost constant over the growth rates studied. RNA concentration per 10^9 cells increased significantly from $\mu = 0.461$ h.$^{-1}$. RNA/DNA ratios reflect this data with respect to growth rate (Fig. 5.7.4.). Below $\mu = 0.461$h.$^{-1}$, the linear relationship appeared to break down. Relatively low cell numbers from samples removed at low growth rates and non-balanced growth were probably the predominant factors. Low cell numbers prevented representative fluorometry of nucleic acid extracts, and consequently, may influence measurements of nucleic acid content at these growth rates.

The relationship between RNA/DNA ratio and growth rate measured by oligonucleotide probing and fluorometry of whole cells (Figs. 5.7.4.) shows a positive correlation. Calibration curves of RNA and DNA concentration determined by oligonucleotide probing (Apps. 5.7.1. and 5.7.2. respectively) were reliable relationships between nucleic acid concentration and pixel sum above background. Concentration of sample nucleic acids in experimental samples can be calculated over a range 0.0125-12μg RNA and 0.65-12.5μg DNA. The linearity of the relationship between RNA/DNA ratio and growth rate μ was statistically significant when analysed with a students t-test. However, differences between successive data points were statistically insignificant and this should be considered when interpreting the relationship between nucleic acid ratios measured by these techniques. The slopes of

RNA/DNA ratios determined by oligonucleotide probing and whole cell fluorometry appeared to be divergent. Whole cell fluorometry is a measurement of total RNA content, whilst oligonucleotide probing is specific to 16S rRNA. The divergence of these slopes is possibly indicative of increased levels of mRNA and tRNA present at higher growth rates. This is supported by Bremer and Dennis (1987), but contradicted by Kjellgaard and Kurland, (1963) who found that soluble RNA concentrations in *E. coli* were constant over a range of growth rates. Clearly, techniques for assessment of both RNA and DNA content are a major factor in determination of nucleic acid ratios under any conditions.

The relationship between *L. monocytogenes* growth rate and nucleic acid ratio determined here and those of a historical data set of 6 organisms were compared in Fig. 5.7.4. (Kerkhof and Ward, 1993 .). This shows that RNA/DNA ratios determined by whole cell fluorometry were in agreement with the theoretical relationship between growth rate and nucleic acid ratio. RNA/DNA ratios determined by oligonucleotide probing are significantly below this theoretical relationship. Slopes of nucleic acid ratio determined by oligonucleotide probing and the historical data set are similar, and suggest either that this method of nucleic acid determination underestimates RNA content, or the relationship between nucleic acid ratio and growth rate for *L. monocytogenes* is different to that shown for six organisms (Kerkhof and Ward, 1993). This historical data set is founded on a limited number of microorganisms and nucleic acid content was assessed by several techniques. Therefore application of this data set is tentative. Any relationship between RNA/DNA ratios suggested for *L. monocytogenes* by these studies would require confirmation employing continuous culture, or balanced growth of the organism at more growth rates.

5.10.3. Effect of HCl addition on *L. monocytogenes* growth and recovery.

Acidification of a stationary phase culture of *L. monocytogenes* in Trivett and Meyer medium to pH 3.0 for 30min. with HCl caused little, if any effect on optical density at 660nm and an insignificant change in colony forming units per ml (Fig. 5.8.1.). Growth has been reported to be inhibited at <pH 4.3 (Farber and Peterkin, 1991) and pH 4.3 (this study) but observations presented here confirm reports that HCl has a minimal effect on *L. monocytogenes* survival (Ita and Hurkins, 1991) when compared to the effect of organic acids, in particular acetic acid at the same pH. Resistance to pH is attributed to efficient maintenance of cytoplasmic pH between 0.5 and 1.5 pH above that of the external environment (Ita and Hurkins, 1991).

When a mid-exponential phase culture of *L. monocytogenes* grown in Trivett and Meyer minimal medium was acidified with HCl to pH 3.0 for 30min, a significant change was observed in colony forming units (Fig. 5.8.3.). However, whilst colony forming units decreased until 7 h. after acid addition before subsequent recovery, a continual decline in optical density at 660nm was observed over the same period. This is probably a reflection on the relative reliability of optical density measurements and viable counts in this study. A decrease in colony forming units suggests that rapidly growing cells are more susceptible to HCl than a stationary phase culture.

Nucleic acid ratios measured by fluorometry and oligonucleotide probing of samples from a stationary phase culture exposed to HCl for 30min. disagree. Whilst both measurements decrease, fluorometric analysis of samples removed after acid addition decreased significantly prior to a slight, but statistically significant rise at the final sample removed. Identical samples assayed by olgonucleotide probing show no trend in any direction after acid addition. This may be due to poor calibration curves (Appendix 5.8.1. and 5.8.2. for RNA and DNA respectivley) which do not permit reliable calculation of experimental nucleic acid concentrations.

Nucleic acid ratios measured prior to acid addition do not show the same pattern as those from *L. monocytogenes* batch culture in a complex medium (Fig.

5.6.2.4. and 5.6.2.5), although samples were removed at only 5, 7.5 and 24 h. culture prior to HCl addition. Whilst growth in Trivett and Meyer medium has been monitored over time (Appendix 5.8.4.), RNA/DNA ratios in a batch culture containing this medium have not been assessed. However, it is anticipated that nucleic acid ratios should exhibit the same trends in batch culture, because growth phases will be the same, particularly if media and culture conditions are designed for the specific organism.

Nucleic acid ratios measured prior to, and following, addition of HCl to a mid-exponential culture of *L. monocytogenes* in Trivett and Meyer medium indicated a significant change in ratio following acid addition (Fig. 5.8.4. and Appendix 5.8.3.). Similar trends were observed by both fluorometry and oligonucleotide probing of nucleic acid extracts. Comparison of nucleic acid data with the colony forming units shows an initial decrease in RNA/DNA ratio which coincides with a decrease observed in colony forming units immediately following acid addition. Nucleic acid ratios increase significantly thereafter until 10 h. after acid addition. Colony forming units increased from 7 h. after acid addition until the final sample point following similar dynamics to batch growth. Trends in nucleic acid ratios in this experiment are more representative of ratios observed in batch culture in earlier studies. These data suggest recovery of colony forming units is preceded by an increase in RNA/DNA ratio, which may indicate an increase in transcription required to facilitate cellular recovery. Given that samples removed will represent an average of cells present, it is possible that cell death may be occurring at a greater rate than cell recovery and/or growth. Therefore, RNA/DNA ratios appear to increase as a group of cells in the sample recover but this is numerically less than the dying population of cells; therefore colony forming units continue to fall.

The effects of HCl on *L. monocytogenes* growth and survival are not as significant as those caused by organic acids, for example acetic acid (Ita and Hurkins, 1991). Acetic acid is commonly used in food production, primarily as a food preservative and it would be more appropriate to examine the effect of this reagent in

future studies. Hydrochloric acid was used in the experiments presented here as a means of sublethally injuring *L. monocytogenes* and monitoring recovery. This reagent was partially successful at causing injury; an increase in the proportion of injured cells would permit better representation of the recovery of the cell population as a whole. However, an ideal experiment to monitor cell recovery after sublethal injury would be to separate the injured cell population from the unaffected population. This presents a considerable challenge and is of limited value to research concerned primarily with reducing food pathogen numbers to a minimum population and preventing recovery.

Examination of the effect of HCl addition on a stationary phase culture of *L. monocytogenes* is probably more representative of the environment where the organism persists. Few organisms will be in the exponential phase of growth in the environment at a given instant and microbial succession on a foodstuff is a rapid process which leads to *L. monocytogenes* creating a microhabitat. Cells will grow until nutrient supply is exhausted, or the surrounding environment is altered to an inhibitory state. Often the food matrix prevents migration of the cell to a fresh area for colonisation. This may be due to physical barriers, or a result of physio-chemical parameters of the foodstuff (Michard and Jardy, 1989; Ryser and Marth, 1987)

5.10.4. Recovery of *L. monocytogenes* after thermal injury.

Exposure of *L .monocytogenes* cultures grown at two temperatures to thermal injury was monitored by measuring optical density and nucleic acid ratios (Figs. 5.9.1. and 5.9.2., respectively). This showed that significant recovery occurred after 24 h. only in the complex medium. Recovery was significantly quicker in an inoculum prepared from *L. monocytogenes* grown at 40°C than from cells grown at 20°C. Addition of 1% (v/v) sodium pyruvate solution did not appear to have any significant effect on the dynamics, or degree of *L. monocytogenes* recovery.

Clearly, any conclusions reached on the basis of these data are founded on a cell population that may contain a significant number of uninjured cells. Enumeration of injured cells may be achieved by use of plating media that contains NaCl, to which heat, or acid injured *L. monocytogenes* cells are sensitive (Bunduki *et. al.*, 1995). Exposure of *L. monocytogenes* to 56°C for 20 min caused substantial injury (84%, Bunduki, *et. al.*, 1995) It is therefore likely that heat exposure used here caused significant injury to the cells. In addition, measurements of nucleic acid content were successful only from fluorometry of whole cells, and are therefore uncorroborated. The value of these heat injury regimes is of doubtful value in the environment. For example, *L. monocytogenes* appears to have altered sensitivity to heat when located intracellularly in bovine polymorphonuclear leukocytes (Doyle *et. al.*, 1987; Farber, 1990).

Studies on heat induced injury of *L. monocytogenes* have concentrated on media constituents and their possible roles in *L. monocytogenes* recovery (Smith, 1990; Smith and Archer, 1988; Smith and Hunter, 1988; Busch and Donelly, 1992). It is proposed that heat injury causes significant disruption of membrane function such that Mg^{2+} ions are lost from cells, which causes ribosomal subunits to dissociate. This renders the ribosomal subunits, in particular the 30S subunit (Kaplan and Apirion, 1973), susceptible to degradation with consequent loss of cellular activity (Busch and Donelly, 1992). Repair from sub-lethal injury involves RNA polymerase, electron transport chain and oxidative phosphorylation mechanisms (Bunduki *et. al.*, 1995) and tentatively, catalase and peroxidase enzymes (Busch and Donnelly, 1992). Consequently, a requirement for divalent cations, in particular Fe^{2+} and Mg^{2+} aids recovery of *L. monocytogenes* cells (Busch and Donnelly, 1992). *L. monocytogenes* contains an efficient membrane bound ferric reductase for ferric ion assimilation (Deneer, *et. al.*, 1995). Sublethal injury may impair the function of this system. The presence of B group vitamins and glucose have been shown to have a stimulatory role in *L. monocytogenes* recovery from injury (Busch and Donnelly, 1992). The medium which successfully recovered heat injured *L. monocytogenes* (TSYGB) contains both

additional yeast extract (a source of B-vitamins) and glucose. Recovery has not been reported in media lacking these supplements, for example tryptone soya broth (Bunduki *et. al.*, 1995). Presence of NaCl in media has been linked to increased thermotolerance and improved recovery of injured *L. monocytogenes* (Anderson *et. al.*, 1991). Minimal media used to monitor recovery in studies by Busch and Donnelly, (1992); Bunduki *et. al.*, (1995) and Anderson *et. al.*, (1991) contain Mg^{2+}, Fe^{2+}, the vitamins B1, B2, lipoic acid and biotin which support growth of *L. monocytogenes* under normal growth conditions but lacks NaCl. However, recovery of heat injured cells is minimal even after 48 hrs at 30°C. This media (Trivett and Meyer, 1971) contains seven amino acids, which have subsequently been shown to be essential for *L. monocytogenes* growth (Premaratne, *et. al.*, 1991), whilst addition of supplementary amino acids, for example glutamine, increased growth rate, but were not essential. A minimal supply of essential amino acids is required, presumably to permit *L. monocytogenes* to synthesise other amino acids required for cellular growth and function. Reactions involved in these pathways will use energy and cellular resources which may be in critical supply after injury and cumulatively overwhelm cellular recovery. These pathways may require ions lost by membrane disruption to function efficiently. An excess supply of amino acids and a ready energy supply (glucose) will aid cellular recovery by removal of this synthetic need.

Decreases in RNA/DNA ratios observed in these studies indicate that *L. monocytogenes* grown at 40°C showed a significantly lower decrease in RNA/DNA ratio upon thermal injury than those grown at 20°C. Recovery of cells grown at 40°C is quicker than those grown at 20°C. After 9 h recovery, cells in TSYGB show a significantly increased RNA/DNA ratio with respect to cells grown at 20°C. Optical density and viable counts indicated similar rates of recovery between cells in TSYGB with, or without 1% (v/v.) pyruvate added. Thermotolerance may be acquired by a cell population if heating rate is gradual. The rate at which this occurs for *L. monocytogenes* has been shown to be $\leq 5°C.min^{-1}$ (Stephens, *et. al.*, 1994). Previous studies indicate that loss of viability in *L. monocytogenes* is linked to the onset of

thermal degradation of 30S ribosomal subunits (Stephens and Jones, 1993). Presence of NaCl in media and prior incubation at elevated growth temperatures (46°C) increases the temperature at which cell viability decreases. This coincides with an increase in 30S ribosomal subunit thermtolerance (Stephens and Jones, 1993).

5.10.5. Relative value of fluorometry and oligonucleotide probing for measurement of RNA/DNA ratios.

In general, RNA/DNA ratios calculated by fluorometry of whole cells, nucleic acid extracts and oligonucleotide probing of nucleic acid extracts show agreement in their trends with respect to time. The ratios differ in value, dependent on the technique used for analysis. For example, nucleic acid ratios observed during the batch growth of *L. monocytogenes* in shake flask culture were greatest when calculated by oligonucleotide probing (RNA/DNA = 19 at 12h.). When *L. monocytogenes* growth rate was varied, maximum nucleic acid ratio was calculated by whole cell fluorometry (RNA/DNA = 9.8 when μ = 1.069 h^{-1}). Theoretical values of nucleic acid ratio calculated for these samples by the equation $RNA/DNA^{theo} = +2 + 5.2\ (\mu)$ (Kerkhof and Ward 1993) are 7.38 and 9.18 for the batch growth in a shake flask and manipulation of growth rate respectively.

Fluorometric analysis of nucleic acid content should if anything, overestimate the amount of RNA present in samples. The technique for RNA quantification (ethidium bromide staining and RNase-mediated fluorescence depletion) will not discriminate between RNA species. At rapid growth rates in *E. coli*, rRNA is estimated to account for 85% of all RNA present (Bremer and Dennis, 1987). Fluorometry will measure all RNA present (presumably mRNA, rRNA and tRNA) and RNA/DNA ratios will be increased. Calibration curves of nucleic acid concentration against fluorescence (Fig. 4.3.1.) show DNA fluorescence is greater than RNA fluorescence per unit mass by a factor of approximately 3.8. This

contradicts the study of LePecq and Paoletti (1966) who state DNA fluorescence is greater by a factor of 2.8. This relationship was based on fluorescence values of calf thymus DNA and yeast transfer RNA. Data presented here are based on the fluorescence of *L. monocytogenes* DNA and *E. coli* 16 and 23S rRNA with respect to concentration. This is at least, of direct relevance to the subsequent studies. However, RNA/DNA ratios calculated throughout are, by definition, 25% greater than those presented by Kerkhof and Ward (1993). This study has attempted to quantify nucleic acid concentrations, as opposed to relative RNA/DNA ratios.

Assessment of RNA/DNA ratios by whole cell fluorometry assumes cells are equally permeable at all phases of culture and that ethidium bromide will have unlimited access into the cell. Pervious studies have shown that significant fluorescence occurs when permeabilised cells were stained with ethidium bromide. This fluorescence was due predominantly to nucleic acids (Fig. 4.3.2.). Ethanol fixation has been used for cell permeabilisation prior to successful analysis of cellular RNA content of Gram-positive bacteria (Roller *et. al.*, 1994). Much RNA is likely to be bound with ribosomal proteins and inaccessible to ethidium bromide. In addition, significant fluorescence may be associated with cellular proteins.

Extensive research has focused on nucleic acid content (in particular rRNA content) in whole cells (Amman *et. al.*, 1995). Success is dependant on cell type, but studies have shown relationships between growth rate and *in-situ* fluorescence of rRNA directed oligonucleotide probes (Poulsen *et. al.*, 1993; Wallner *et. al.*, 1993). Fluorometry of nucleic acid extracts removes problems associated with cell permeability and nucleic acid availability but problems are encountered due to variability of nucleic acid extraction efficiency. This problem has been addressed by Kramer and Singleton (1992) and Kerkhof and Ward (1993), and the study reported here estimated nucleic acid extraction effiency is to be 40% by the method used (Chapter 4). When *L. monocytogenes* was grown in a non-buffered complex medium nucleic acid bands are seen to become degraded at the onset of death phases (Fig. 5.6.1.2.). Although this is a useful indicator of *L. monocytogenes* viability in this

example, fluorometric response may be altered. This is possibly due to relaxation of both DNA and rRNA secondary structure upon partial degradation permitting increased ethidium bromide intercalation. Implications of partial nucleic acid degradation upon quantitative oligonucleotides probing are unclear. Annealing of oligonucleotide probe may be inhibited by the presence of significant amounts of sheared nucleic acids in a similar fashion to use of ultrasonicated DNA to prevent non-specific probe binding in some nucleic acid hybridisation protocols (Sambrook *et. al.*, 1982).

Analysis of nucleic acid content by oligonucleotide probing depends on a reliable standard relationship between signal intensity and nucleic acid concentration. In most examples presented in this study, the equations describing standard curves of signal intensity and nucleic acid concentration were statistically valid and confidence could be placed in nucleic acid concentrations derived from them. However, calculation of DNA concentration will be susceptible to error. The proportion of DNA that is potential target for the oligonucleotide probe used here is 0.004% of total DNA (assuming there are 6 copies of the 16S rRNA gene), whilst the percentage target of RNA is 0.44% target sequence to the oligonucleotide probe. Consequently, greater amounts of DNA must be applied to the membrane to result in an equivalent signal to RNA. Error in DNA concentration calculation will be greatest at the lowest signals produced. Maximum deviation is shown between practical and mathematical relationships at low DNA concentration. Membranes may become saturated with DNA before reliable calculation of DNA concentration can be achieved. Therefore, oligonucleotide estimation of nucleic acid content may underestimate DNA concentration and ultimately overestimate RNA/DNA ratios. Data presented here supports this hypothesis for most experiments, but significantly, nucleic acid ratios calculated by oligonucleotide probing when *L. monocytogenes* growth rate is varied are less than those calculated by whole cell fluorometry. These findings contradict those of Kerkhof and Ward (1993) which indicates an underestimation of rRNA concentration when measured by oligonucleotide probing. Explanations offered are

that rRNA secondary structure was not completely denatured, contaminating ribosomal proteins remained, RNA was lost during filter hybridisation and that nucleic acid probe binding (200bp. in length) was inhibited. In these studies, RNA was denatured at a higher temperature (68°C) than by Kerkhof and Ward (1992) and a 21bp. oligonucleotide probe was used throughout. The domain to which the probe hybridised has not been reported to be inhibitory for probe annealing.

5.11. CONCLUSIONS.

- RNA/DNA ratios in batch culture increase rapidly to mid-exponential phase and subsequently decrease rapidly.

- Nucleic acids may be detected in significant quantities despite low, or insignificant viable count data.

- *L. monocytogenes* may survive in significant numbers for extended periods in one culture if pH is maintained.

- Nucleic acid ratios fall rapidly following exponential growth phase; further decreases are gradual.

- Addition of HCl to a stationary, and mid-exponential culture of *L. monocytogenes* in minimal medium has little effect on cell numbers. Changes in RNA/DNA ratios following acid addition do not exhibit a discernible trend.

- Culture temperature prior to sublethal heat injury has significant effect on recovery time of *L. monocytogenes*.

- Recovery of heat-injured *L. monocytogenes* occurs in complex medium, but not in a minimal medium.

- Addition of pyruvate has little, or no effect on extent, or rapidity of recovery in these media.

CHAPTER 6.

NUCLEIC ACID SEQUENCE ANALYSIS OF *Listeria spp.*

6.1. Aims.

- To examine 16S rDNA sequences of *Listeria spp.* amplified by PCR primers MV2/MV9R.

- To assess 16S rDNA sequence divergence amongst culture collection strains and patient isolates.

6.2. Materials and Methods.

6.2.1. DNA Sequencing.

DNA from strains of *Listeria* spp. (Table 6.1) were PCR amplified in quintuplicate series with the 'universal' primers pA and pH' (Edwards *et. al.*, 1989, Table 3.2.1.), following the same procedure described in section 3.2.3. Products (12µl aliquots) were visualised on agarose gels and if the desired product band was present, products from replicate tubes were pooled and purified with Centricon-100 tubes (Amicon. Inc., Beverly, N.J., USA.). Briefly, PCR products were loaded into Centricon tubes and 2ml sterile ultra-pure water added. Tubes were centrifuged for 20min. at 400g and a further 2ml water added. This procedure was repeated a total of 3 times. Following washing steps with ultrapure water, Centricon tubes were inverted, and 50µl sterile ultra-pure water added to the central well to aid DNA elution from the columns. Cleaned PCR products were eluted by centrifugation at 350g for 5min. Amplified DNA samples were concentrated by addition of 0.1 volume 4.5M sodium

Table 6.1. *Listeria* spp. subject to nucleic acid sequencing.

Species	Strain No.
L. monocytogenes	19113
	19116
	1005
	1800
	2073
	10999
L. innocua	33090
	1909
	2393
L. ivanovii	11846
L. seeligeri	35967

N.B. Further details concerning strain history and serotype are available in Table 2.1.

acetate (pH5.3) and 2 volumes 95% (v/v) ethanol and DNA precipitated for a minimum of 2h. at -20°C. DNA was pelleted by centrifugation at 9440g for 30min. Pellets were briefly washed in 70% (v/v) ethanol, centrifuged for a further 15min. at 9440g and resuspended in 10μl sterile ultra-pure water. Products (1μl aliquots: 1ml water) were compared on 1%(w/v) agarose gels with reference to a titration series of λ -phage DNA of known concentration and diluted in ultra pure water as required to 500ng.μl^{-1}.

To examine DNA sequences targeted by oligonucleotides MV2 and MV9R, PCR primers tentatively diagnostic for *L. monocytogenes* (see Section 3.2.1. and Table 3.2.1. for further details). Automated Taq DyeDeoxy™ terminator cycle sequencing (Applied Biosystems Model 373A DNA sequencer) was performed in both 5'→3' and 3'→5' direction with primers universal primers pB and pD' (region complementary to MV2) and pF and pG' (complimentary to primer MV9R; Edwards *et. al.*, 1989; Table 3.2.1.).

6.2.2. DNA sequence analysis.

Nucleic acid sequence traces were initially examined visually for insertions and errors. Complementary forward and reverse sequences were compared by pair-wise analysis using the Wisconsin Genetics Computer Group package (GCG) version 8.0 (Devereux *et. al.*, 1984). Mismatches between complimentary sequence pairs in raw data were re-analysed before further analysis. Experimental sequences were aligned with PILEUP using the GCG package (Devereux *et. al.*, 1984). This permitted differences and errors in experimental data to be examined. Ambiguous or nonsensical data, occurring particularly at extreme ends of the sequences were ignored.

Nucleotide differences between experimentally determined sequences and *L. monocytogenes* and *L. innocua* sequences from the EMBL database (reference sequences) were compared. Reference sequences were imported from the EMBL

database into the GCG package and aligned with experimental data by PILEUP analysis as described above. Regions corresponding to the annealing sites of primers MV2 and MV9R were compared visually and any differences noted.

Further DNA sequence analysis was performed using the genetic data environment sequence editor (GDE) version 2.2 (Eisen *et. al.*, 1992). Experimentally derived sequences were imported into GDE and aligned to 16S rRNA sequences from *Listeria spp., Brochothrix campestris, Bacillus thurigiensis, Carnobacterium alterfunditum, C. gallinarum* and *Lactococcus lactis*. These sequences were derived from low G + C content bacteria previously shown to have a close phylogenetic relationship to *Listeria spp.* (Collins *et. al.*, 1991). Phylogenetics relationships were initially determined on database derived sequences only. Nucleotide positions for analysis were selectively scored according to Lane (1991). This 'mask' examines nucleotide positions which are of phylogenetic and discriminatory value, whilst ignoring those of limited value, particularly in 'hypervariable regions'. Phylogenetic trees were constructed from distance matrix data (calculated by the algorithm of Jukes and Cantor 1969) by the least squares methods of DeSoete (1983) using the Treetool package (Maciukenas 1991).

Experimental sequences were aligned to the mask of Lane (1991). Those nucleotide positions which corresponded to both experimental sequence and analytical mask were examined. Nucleotide positions of ambiguous identity were excluded from analysis. In all investigations, masks designed to analyse experimental data were initially tested by examination of relationships between reference DNA sequences prior to inclusion of experimental DNA sequences. Distance matrix analysis was used to construct phylogenetic trees on the basis of either a mask targeting either pB/pD' data or pF/pG' datasets, as appropriate. Finally, phylogenetic trees were reconstructed from distance matrix analysis of combined data produced from both DNA fragments (673 nucleotides).

6.3. Results.

Sequence information which covered the V2 area of the 16S rRNA gene (143-220bp) was obtained with both primers pB and pD' for strains 33090, 1909, 35967 and 10815 only. Partial sequence information was obtained from this primer combination from strains 19113, 19116, 2073, and 1800. Sequence data covering the V9 region of 16S rRNA gene (1215-1297bp) was obtained with primers pF/pG' for the following: 1005, 1800, 2073, 10999, 19113, 19116, 1909, 2393, 33090, and 11846.

Comparison of these sequences with those of *L. monocytogenes* and *L. innocua* from the database where PCR primers MV2 and MV9R annealed showed differences in both regions (Fig. 6.3.1. and Fig. 6.3.2. respectively). Whilst *L. innocua* strain ATCC 33090 appeared to have an identical sequence to that of the reference *L. innocua* strain in both regions examined, a clinical isolate (*L. innocua* 1909) showed 3 nucleotide differences to the reference *L. innocua* sequence in the region 220-230bp. Consequently, sequence information derived for *L. innocua* strains showed one and three nucleotide differences, respectively between *L. innocua* strains 33090 and 1909, where PCR primer MV2 anneals (206-225bp).

Differences were seen between *L. monocytogenes* sequences from clinical isolates 1800 and 2073 in the region 1317-1337bp and the reference sequences. These isolates showed two nucleotide differences in the region where PCR primer MV9R is targeted (1317-1337bp). However, no differences were observed in this region between experimentally derived *L. innocua* sequences and that of the reference sequence. Therefore, one nucleotide difference is recorded between PCR primer MV9R and *L. innocua* strains sequenced.

Relationships between DNA sequences were explored by distance matrix analysis and phenograms reconstructed using the Treetool package.

Analysis of sequences produced from primers pB/pD' showed that with the exception of *L. monocytogenes* strain 19113, all experimentally derived sequences

```
                    51                                                  100
L. seeligeri 35967  NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNN NNTTTNAGGC AANTCATTGA
L. innocua 33090    NNNNNNNNNNN NNNNNNNNNNN NNNNNNNNNTT TTNGCTANAC ATGCAAGTCG
L. innocua 1909     ...NNNNNNN NNNNNNNNNNN NGNNNACGNG NGCCTANAAC ATGCAAGTCG
L. monocytogenes    .......CU CAGGACGAAC GCUGGCGGCG UGCCUAAUAC AUGCAAGUCG
L. innocua          GAUCCUGGCU CAGGACGAAC GCUGGCGGCG UGCCUAAUAC AUGCAAGUCG
L. grayi 10815      NNNNNNNNNNN NNNNNNNNNNN NNNNNNTTTTT NGGCTAANAA ATGCAATTCG

                    101                                                 150
L. seeligeri 35967  AACGAACG.G AGGAAGAGTT GTTTTTTCCA AAAGTTAGTG GCGGACGGGT
L. innocua 33090    AACGAACG.G AGGAAGAGCT TGTTTTTTCC AAAGTTAGTG GCGGACGGGT
L. innocua 1909     AACGAACG.G AGGAAGAGC. TGTTCTT.CC AATGTTAGTG GCGGACGGGT
L. monocytogenes    AACGAACG.G AGGAAGAGCU UGCUCUU.CC AAAGUUAGUG GCGGACGGGU
L. innocua          AACGAACG.G AGGAAGAGCU UGCUCUU.CC AAAGUUAGUG GCGGACGGGU
L. grayi 10815      AANGAATGAC CTTAGGAGCT TGCTCCTTTG TTCGTTAGTG GCGGACGGGT

                    151                                                 200
L. seeligeri 35967  GAGTAANAAC GTGGGCAACC TGCCTGTAAG TTGGGGATAA TTCCGGGAAA
L. innocua 33090    GAGTAAACAC GTGGGCAACC TGCCTGTAAG TTGGGGATAA CTCCGGGAAA
L. innocua 1909     GAGTAAACAC GTGGGCAACC TGCCTGTAAG TTGGGGATAA CTCCGGGAAA
L. monocytogenes    GAGU.AACAC GUGGGCAACC UGCCUGUAAG UUGGGGAUAA CUCCGGGAAA
L. innocua          GAGU.AACAC GUGGGCAACC UGCCUGUAAG UUGGGGAUAA CUCCGGGAAA
L. grayi 10815      GAGTAAACAC GTGGGCAACC TGCCTGTAAG ATTGGGATAA CTCCGGGAAA

                    201                                                 250
L. seeligeri 35967  CCGGGGCTAA TACCGAATGA TAAGGAGTGA CGCATGTCAC TGGCTTTGAA
L. innocua 33090    CCGGGGCTAA TACCGAATGA TAGAGTGTGG CGCATGCCCA CGCTCTTGAA
L. innocua 1909     CCGGGGCTAA TACCGAATGA TAAGATGTGG CGCATG.CCA CGCTTTTGAA
L. monocytogenes    CCGGGGCUAA UACCGAAUGA UAAAGUGUGG CGCAUG.CCA CGCUUUUGAA
L. innocua          CCGGGGCUAA UACCGAAUGA UAGAGUGUGG CGCAUG.CCA CGCUCUUGAA
L. grayi 10815      CCGGAGCTAA TACCGAATAA TAATCAGCTC CGCATGGAGC AGGT.TTGAA

                    251                                                 300
L. seeligeri 35967  AGATGGTTTC GGCTATCGCT TACAGATGGG CCCGCGGTGC ATTAGCTAGT
L. innocua 33090    AGATGGTTTC GGCTATCGCT TACAGATGGG CCCGCGGTGC ATTAGCTAGT
L. innocua 1909     AGATGGTTTC GGCTATCGCT TACAGATGGG CCCGCGGTGC ATTAGCTAGT
L. monocytogenes    AGAUGGUUUC GGCUAUCGCU UACAGAUGGG CCCGCGGUGC AUUAGCUAGU
L. innocua          AGAUGGUUUC .GCUAUCGCU UACAGAUGGG CCCGCGGUGC AUUAGCUAGU
L. grayi 10815      AGGCGGCTTC GGCTGTCACT TACAGATGGG CCCGCGGTGC ATTAGCTAGT

                    301                                                 350
L. seeligeri 35967  TGGTAGGGTA AAGGCCTACC AAGGCAACGA TGCATAGCCG ACCTGAGAGG
L. innocua 33090    TGGTAGGGTA ATGGCCTACC AAGGCAACGA TGCATAGCCG ACCTGAGAGG
L. innocua 1909     TGGTAGGGTA ATGGCCTACC AAGGCAACGA TGCATAGCCG ACCTGAGAGG
L. monocytogenes    UGGUAGGGUA AUGGCCUACC AAGGCAACGA UGCAUAGCCG ACCUGAGAGG
L. innocua          UGGUAGGGUA AUGGCCUACC AAGGCAACGA UGCAUAGCCG ACCUGAGAGG
L. grayi 10815      TGGTGGGGTA AAGGCCTACC AAGGCGACGA TGCATAGCCG ACCTGAGAGG
```

Figure 6.3.1. Pileup analysis of nucleotide sequences obtained from experimental strains covering the region targeted by PCR primer MV2 (206-225nt.). Differences between sequences in regions analysed by distance matrix methods shown thus: •.

```
                    351                                                 400
L. seeligeri 35967  GTGATCGGCC ACACTGGGAC TGAGACACGG CCCAGACTCC TACGGGAGGC
L. innocua 33090    GTGATCGGCC ACACTGGGAC TGAGACACGG CCCAGACTCC TACGGGAGGC
L. innocua 1909     GTGATCGGCC ACACTGGGAC TGAGACACGG CCCAGACTCC TACGGGAGGC
L. monocytogenes    GUGAUCGGCC ACACUGGGAC UGAGACACGG CCCAGACUCC UACGGGAGGC
L. innocua          GUGAUCGGCC ACACUGGGAC UGAGACACGG CCCAGACUCC UACGGGAGGC
L. grayi 10815      GTGATCGGCC ACACTGGGAC TGAGACACGG CCCAGACTCC TACGGGAGGC

                    401                                                 450
L. seeligeri 35967  AGCAGTAGGG AATCTTCCGC AATGGACGAA AGTCTGACGG AGCAACGCCG
L. innocua 33090    AGCAGTAGGG AATCTTCCGC AATGGACGAA AGTCTGACGG AGCAACGCCG
L. innocua 1909     AGCAGTAGGG AATCTTCCGC AATGGACGAA AGTCTGACGG AGCAACGCCG
L. monocytogenes    AGCAGUAGGG AAUCUUCCGC AAUGGACGAA AGUCUGACGG AGCAACGCCG
L. innocua          AGCAGUAGGG AAUCUUCCGC AAUGGACGAA AGUCUGACGG AGCAACGCCG
L. grayi 10815      AGCAGTAGGG AATCTTCCGC AATGGACGAA AGTCTGACGG AGCAACGCCG

                    451                                                 500
L. seeligeri 35967  CGTGTATGAA NGAGGTTTTC GGATCGTAAA GTACTGTTGT TAGAG.AAGA
L. innocua 33090    CGTGTATGAA NGAGGTTTTC GGATCGTAAA GTACTGTTGT TAGAG.AAGA
L. innocua 1909     CGTGTATGAA GAAGGTTTTC GGATCGTAAA GTACTGTTGT TAGAG.AAGA
L. monocytogenes    CGUGUAUGAA GAAGGUUUUC GGAUCGUAAA GUACUGUUGU UAGAG.AAGA
L. innocua          CGUGUAUGAA GAAGGUUUUC GGAUCGUAAA GUACUGUGGU UAGAG.AAGA
L. grayi 10815      CGTGTGTGAA NNAGGTTTTC GGATCGTAAA GCACTGTTGT TAGAG.AAGA

                    501                                                 550
L. seeligeri 35967  ACAAGGATAA GAGTAACTGC TTGTCCCTTG ACGGTATCTA ACCAGAAAGC
L. innocua 33090    ACAAGGATAA GAGTAANTGN TTGT.CCTTG ACGGTATCTA ACCNNCCCCC
L. innocua 1909     ACAAGGATAA GAGTAACTGC TTGNCCTTTG ACGGGCCCTN ACCATTAAAA
L. monocytogenes    ACAAGGAUAA GAGUAACUGC UUGUCCCUUG ACGGUAUCUA ACCAGAAAGC
L. innocua          ACAAGGAUAA GAGUAACUGC UUGUCCCUUG ACGGUAUCUA ACCAGAAAGC
L. grayi 10815      ACAAGGATAA GAGTAACTGC TTGTCCCTTG ACGGTATCTA ACCAGAAAGC
```

Figure 6.3.1. (continued).

```
                    1151                                                1200
L. innocua          GCGCAACCCU UGAUUUUAGU UGCCAGCAUU UAGUUGGGCA CUCUAAAGUG
L. monocytogenes    GCGCAACCCU UGAUUUUAGU UGCCAGCAUU UAGUUGGGCA CUCUAAAGUG
L. innocua 1909     GCGCAACCCT TGATTTTAGT TGCCAGCATT CAGTTGGGCA CTCTAAAGTG
L. monocytogenes 1800 GCGCAACCCT TGATTTTAGT TGCCAGCATT CAGTTGGGCA CTCTAAAGTG
L. innocua 33090    GCGNAACCCT TGATTTTAGT TGCCAGCATT TAGTTGGGCA CTCTAAAGTG
L. innocua 2393     GCGAACCCTT GTATTTTAGT TGCCAGCATT TAGTT.GGCA CTCTAAAGTG
L. monocytogenes 2073 ......AACC TNGATTNNGT NGNCAGNATT TAGTTGGGNA CTCTAAAGTG

                    1201                                                1250
L. innocua          ACUGCCGGUG CAAGCCGGAG GAAGGUGGGG AUGACGUCAA AUCAUCAUGC
L. monocytogenes    ACUGCCGGUG CAAGCC.GAG GAAGGUGGGG AUGACGUCAA AUCAUCAUGC
L. innocua 1909     ACTGCCGGTG CAAGCCGGAG GAAGGTGGGG ATGACGTCAA ATCATCATGC
L. monocytogenes 1800 ACTGCCGGTG CAAGCCGGAG GAAGGTGGGG ATGACGTCAA ATCATCATGC
L. innocua 33090    ACTGCCGGTG CAAGCCGGAG GAAGGTGGGG ATGACGTCAA ATCATCATGC
L. innocua 2393     ACTGCCGGTG CAAGCCGGAG GAAGGTGGGG ATGACGTCAA ATCATCATGC
L. monocytogenes 2073 ACTGCCGGTG CAAGCCGGAG GAAGGTGGGG ATGACGTCAA ATCATCATGC

                    1251                                                1300
L. innocua          CCCUUAUGAC CUGGGCUACA CACGUGCUAC AAUGGAUGGU ACAAAGGGUC
L. monocytogenes    CCCUUAUGAC CUGGGCUACA CACGUGCUAC AAUGGAUAGU ACAAAGGGUC
L. innocua 1909     CCCTTATGAC CTGGGCTACA CACGTGCTAC AATGGATAGT ACAAAGGGTC
L. monocytogenes 1800 CCCTTATGAC CTGGGCTACA CACGTGCTAC AATGGATAGT ACAAAGGGTC
L. innocua 33090    CCCTTATGAC CTGGGCTACA CACGTGCTAC AATGGATGGT ACAAAGGGTC
L. innocua 2393     CCCTTATGAC CTGGGCTACA CACGTGCTAC AATGGATGGT A.AAAGGGTC
L. monocytogenes 2073 CCCTTATGAC CTGGGCTACA CACGTGCTAC AATGGATAGT ACAAAGGGNC

                    1301                                                1350
L. innocua          GCGAAGCCGC GAGGUGGAGC CAAUCCCAUA AAACCAUUCU CAGUUCGGAU
L. monocytogenes    GCGAAGCCGC GAGGUGGAGC UAAUCCCAUA AAACUAUUCU CAGUUCGGAU
L. innocua 1909     GCGAAGCCGC GAGGTGGAGC CAATCCCATA AAACCATTCT CAGTTCGGAT
L. monocytogenes 1800 GCGAAGCCGC GAGGTGGAGC CAATCCCATA AAACCATTCT CAGTTCGGAT
L. innocua 33090    GCGAAGCCGC GAGGTGGAGC CAATCCCATA AAACCATTCT CAGTTCGGAT
L. innocua 2393     GCGAAGCCGC GAGGTGGAGC CAATCCCATA AAACCATTCT CAGTTCGGAT
L. monocytogenes 2073 GCGAAGCCGC GAGGTGGAGC TAATCCCATA AATCTATTCT CAGTCCGGAT

                    1351                                                1400
L. innocua          UGUAGGCUGC AACUCGCCUn CAUGAAGCCG GAAUCGCUnG UAAUCGUGGA
L. monocytogenes    UGUAGGCUGC AACUCGCCUA CAUGAAGCCG GAAUCGCUAG UAAUCGUGGA
L. innocua 1909     TGTAGGCTGC AACTCGCCTA CATGAAGCCG GAATCGCTAG TAATCGTGGA
L. monocytogenes 1800 TGTAGGCTGC AACTCGCCTA CATGAAGCCG GAATCGCTAG TAATCGTGGA
L. innocua 33090    TGTAGGCTGC AACTCGCCTA CATGAAGCCG GAATCGCTAG TAATCGTGGA
L. innocua 2393     TGTAGGCTGC AACTCGCCTA CATGAAGCCG GAATCGCTAG TAATCGTGGA
L. monocytogenes 2073 NGTNGGNTGC AACTCGCCTA CATGAAGCCG GAATCGCTAG TCATCGTGGA

                    1401                                                1450
L. innocua          UCAGCAUGCC ACGGU.nAAU ACGUUCCCGG GCCUnGUACA CACCGCnCGU
L. monocytogenes    UCAGCAUGCC ACGGU.GAGU ACGUUCCCGG GCCUUGUACA CACCGCCCGU
L. innocua 1909     TCAGCATGCC ACGGT.GAAT ACGTCCCCGG GCCTTGTACA CACCGCCCGT
L. monocytogenes 1800 TCAGCATGCC ACGGTGGAAT ACGTCCCCGG GCCTTGTTCA CACCGCCCGT
L. innocua 33090    TCAGCATGCC ACGGT.GAAT ACGTCCCCGG GCCTTGT..A CACGGCCCGT
L. innocua 2393     TCAGCATGCC ACGGT.GAAT ACGTCCCCGG GCCTTGTACA CACCGCCCGN
L. monocytogenes 2073 TCAGCATGTC ACGGTGTANT ACGTTCCCCG GNCCTNGTTC CACACCCNNT
```

Figure 6.3.2. Pileup analysis of nucleotide sequences from experimental strains convering the region targeted by PCR primer MV9R (1317-1337nt.). Differences between sequences shown thus:•.

were of greater similarity to each other than to *Listeria* spp. reference sequences (Fig. 6.3.3.). Of note, is the close association of *L. monocytogenes* and *L. welshimeri* reference sequences. *L. innocua* is just located on a separate branch as determined by this analysis. Distance matrix analysis of *Listeria* spp. reference sequences in the region produced by primers pF/pG' significantly separate *L. monocytogenes* and *L. innocua* sequences. By this method of analysis, *L. monocytogenes* was located on a distinct branch, whilst *L. innocua* showed greatest similarity to *L. seeligeri* and *L. welshimeri*, respectively. Addition of experimentally-derived sequence information to this analysis produced further grouping of *Listeria* spp. strains (Fig. 6.3.4.). All *L. monocytogenes* strains successfully sequenced showed most similarity to the reference sequence of *L. monocytogenes*; both *L. innocua* and *L. ivanovii* sequences showed greatest similarity to the reference sequences of *L. innocua* and *L. ivanovii*, respectively (Fig. 6.3.4.). When both fragments were analysed together, *L. monocytogenes* and *L. innocua* reference strains were grouped together (data not shown). Addition of experimental sequence information showed that with the exception of *L. monocytogenes* strain 2073, experimental *L. monocytogenes* and *L. innocua* strains grouped closest to the respective reference sequences (Fig. 6.3.5.).

Finally, members of the genus *Listeria* and other low G+C content Gram-positive bacteria were included in distance matrix analysis of the two sequence fragments (Fig. 6.3.6.). This indicated the close relationship between members of the genus *Listeria* and showed a close relationship between *L. monocytogenes* and *L. innocua*. Addition of experimental sequence information in this analysis suggested that these sequences have closest relationship to *L. monocytogenes* and *L. innocua* reference sequences (Fig. 6.3.7.), although the discrimination of *L. monocytogenes* and *L. innocua* strains sequenced was indistinct.

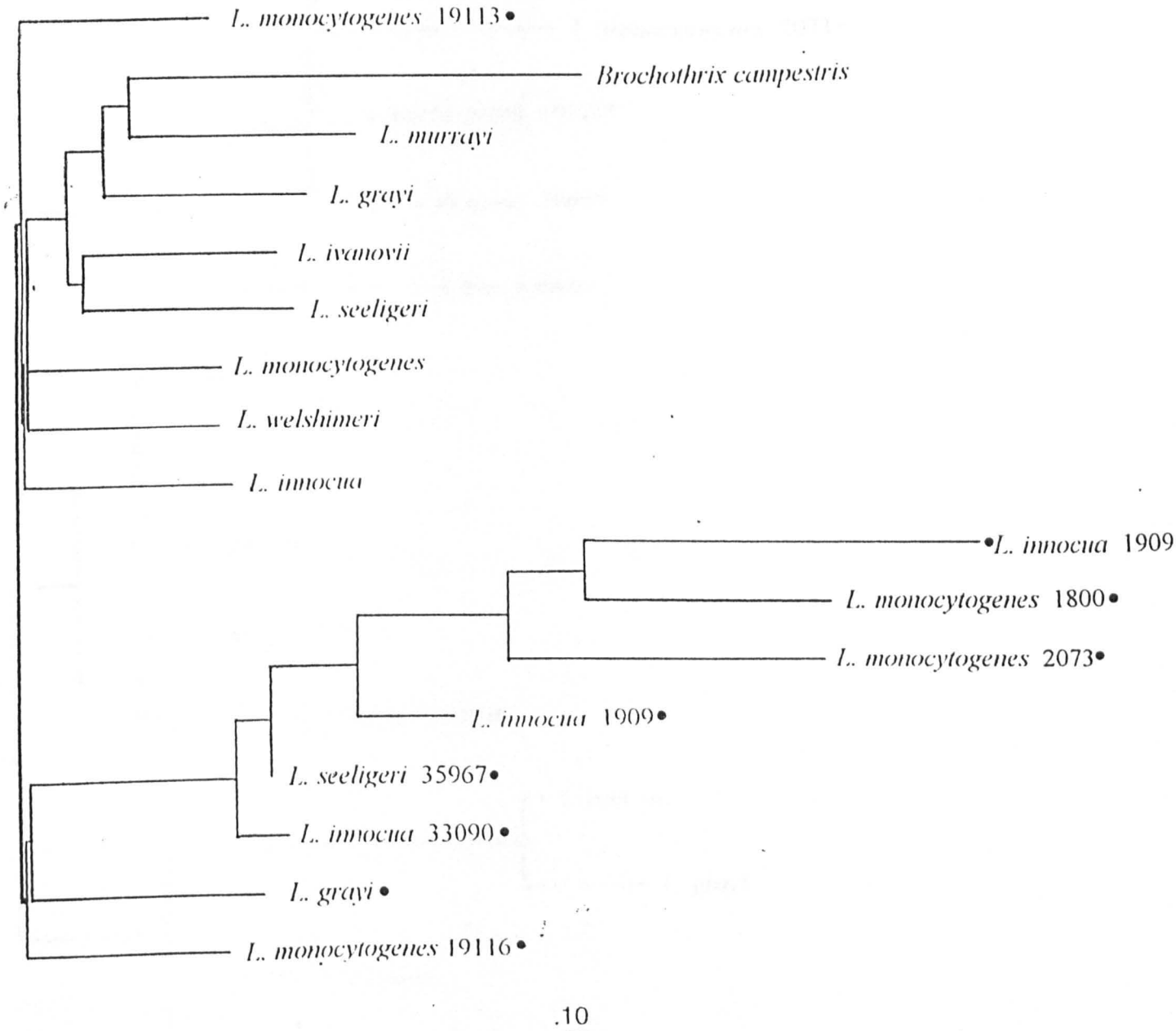


Figure 6.3.3. Distance matrix analysis of 362 nucleotide positions from experimental and database sequences encompassing the region targeted by PCR primer MV2. Scale bar represents nucleotide changes per position.

Experimental sequences denoted thus: •

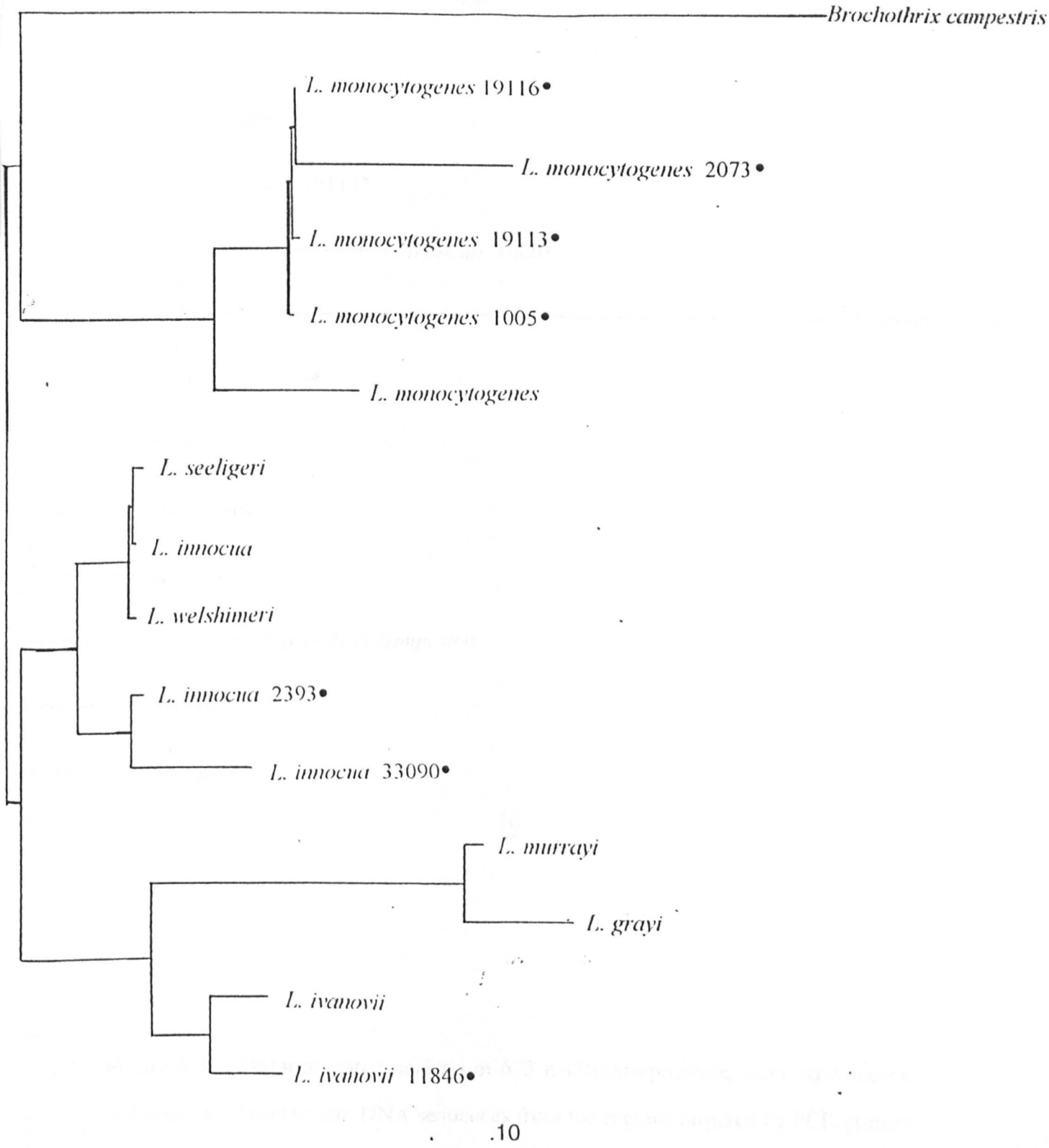


Figure 6.3.4. Distance matrix analysis of 311 nucleotide positions from experimental and reference *Listeria spp.* DNA sequences from the region targeted by PCR primer MV9R. Scale bar represents nucleotide changes per position. Experimental sequences denoted thus: •.

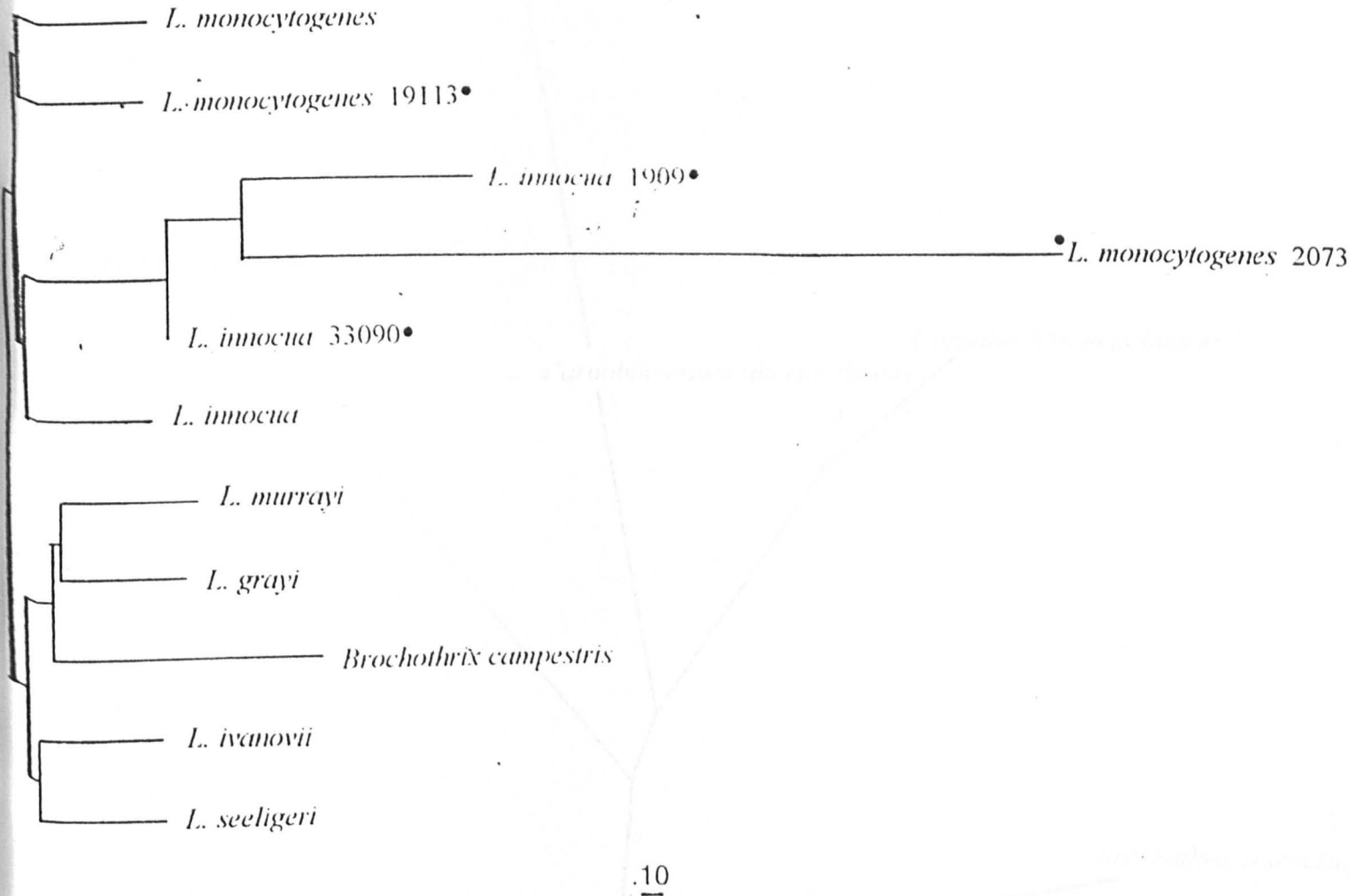


Figure 6.3.5. Distance matrix analysis of 673 nucleotide positions from experimental and reference *Listeria spp*. DNA sequences from the regions targeted by PCR primers MV2/MV9R. Scale bar represents nucleotide changes per position. Experimental sequences denoted thus: •.

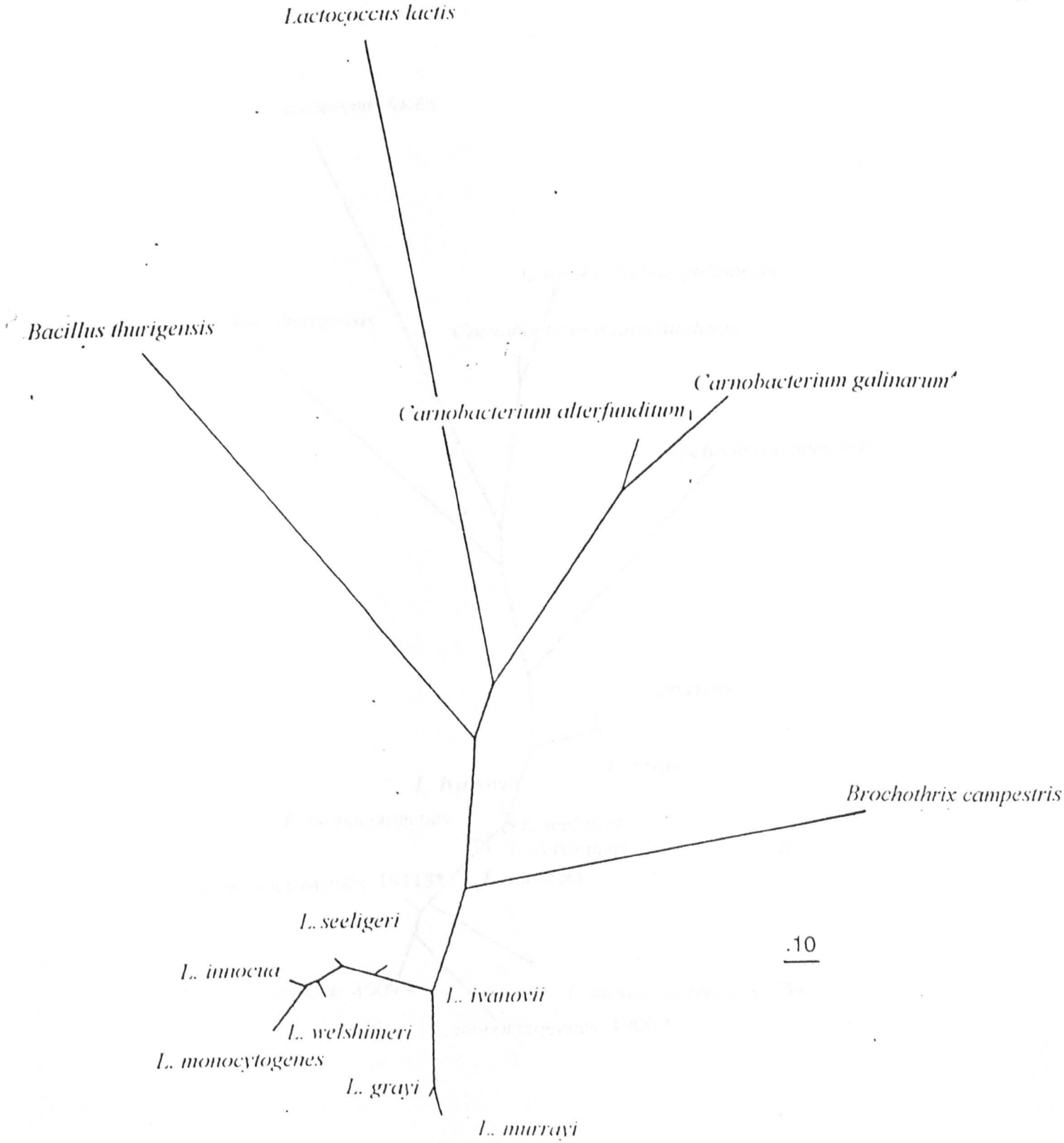


Figure 6.3.6. Unrooted phylogenetic tree, or network of distance matrix analysis of 673 nucleotide positions from reference DNA sequences of representative low G + C content bacteria. Scale bar denotes nucleotide changes per position.

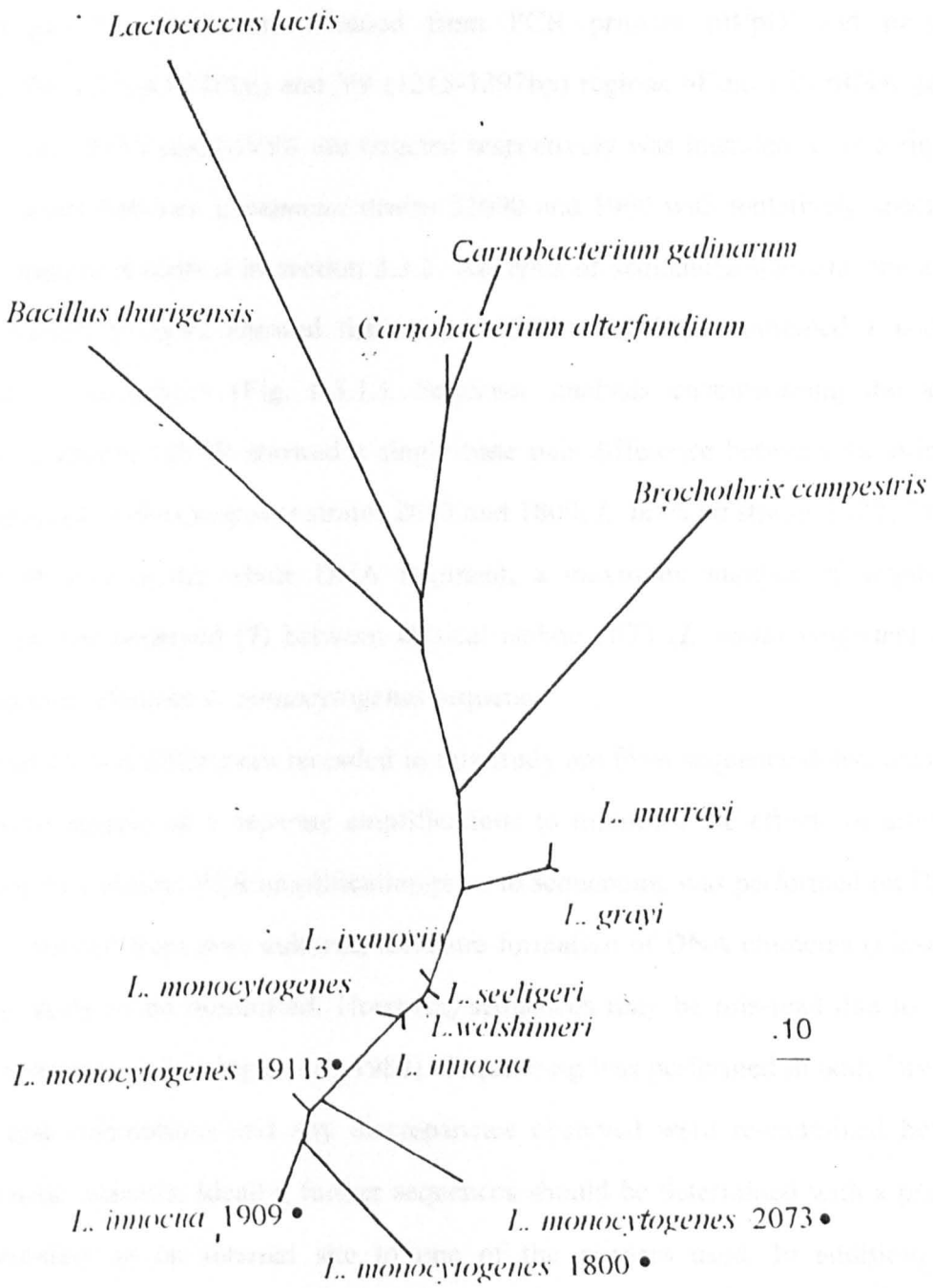


Figure. 6.3.7. Unrooted phylogenetic tree, or network of distance matrix analysis of 673 nucleotide positions from experimental *Listeria spp.* DNA and reference DNA sequences. Experimental sequences are shown thus: •. Scale bar denotes nucleotide changes per position.

6.4. Discussion.

Sequence information obtained from PCR primers pB/pD' and pF/pG' spanning the V2 (143-220bp) and V9 (1215-1297bp) regions of the 16S rRNA gene where primers MV2 and MV9R are targeted respectively was intended to investigate cross-reactivity between *L. innocua* strains 33090 and 1909 with tentatively species-specific primers described in section 3.3.2. Analysis of sequences spanning the area complementary to MV2 showed that strains 33090 and 1909 contained 1 and 2 differences, respectively (Fig. 6.3.1.). Sequence analysis encompassing the area targeted by primer MV9R showed a single base pair difference between the primer sequence and *L. monocytogenes* strains 2073 and 1800, *L. innocua* strains 1909, 2393, and 33090. Across the whole DNA fragment, a maximum number of sequence differences was observed (7) between clinical isolate 2073 (*L. monocytogenes*) and the respective reference *L. monocytogenes* sequence.

Nucleotide differences recorded in this study are from sequence determination of a pooled sample of 5 separate amplifications to minimise the effects of artefact formation. In addition, PCR amplification prior to sequencing was performed on DNA extracts obtained from pure cultures, therefore formation of DNA chimeras (Liesack, 1991) is likely to be minimised. However, sequences may be mis-read due to Taq polymerase errors (Dunning *et. al.*, 1988). Sequencing was performed in both forward and reverse orientations and any discrepancies observed were re-examined before phylogenetic analysis. Ideally, further sequences should be determined with a primer complimentary to an internal site to one of the primers used. In addition, the fragments produced are not contiguous and further sequence determination should include this area of DNA.

These data substantiate the difficulty encountered (section 3.2.3.) in exclusive detection of *L. monocytogenes* with PCR primers targeting V2 and V9 regions of 16S rRNA. The number of base pair mis-matches where primers MV2/MV9R anneal probably contribute to cross-reactivity observed between *L. innocua* strains. Whilst *L.*

innocua 33090 contains a total of 2 differences and was consistently amplified, strain 1909 demonstrated a total of three nucleotide mis-matches in the region where these primers anneal and was not amplified at temperatures above 54°C. A single base pair mismatch in an oligonucleotide primer:target duplex is sufficient to cause a significant temperature decrease in the melting point of the duplex (Wallace *et. al.*, 1979). Therefore, the sequence differences shown here and cross-reactivity of tentatively *L. monocytogenes* specific PCR primers require further investigation to determine conclusively the cause of this phenomenon. Oligonucleotide probing with probe MV9RP2 (see Table 3.2.1.) demonstrated cross reactivity with *L. innocua* strains 33090, 1909 and 2393 at hybridisation temperatures above 45°C (Table 3.3.2.). When PCR fragments were hybridised with oligonucleotide probes MV9P and MV9RP2 cross reactivity was observed with PCR fragments derived from *L. innocua* 33090 (Fig. 3.3.2.i.).

Difficulty in identification of *L. monocytogenes* exclusively on the basis of 16S rRNA sequence alone has been demonstrated by Czajka *et. al.*, (1993). This study screened a variety of *L. monocytogenes* serotypes and showed by RAPD analysis that some strains and/or serotypes of *L. innocua* and *L. monocytogenes* may posses identical nucleotide sequences in the V2 region (143-220bp) where primer MV2 is targeted. Nucleotide differences observed in the V9 region (1215-1297bp) where primer MV9R is targeted were reported to be consistent between serotypes of *L. monocytogenes* and single example of *L. innocua* examined. Therefore sequence signatures for *L. monocytogenes* were unsuitable in the V2 region of the 16S rRNA gene, but stable sequence signatures were shown in the V9 region of the gene. Sequencing of a 1458bp. 16S rRNA fragment derived from a *L. innocua* and *L. monocytogenes* strain showed 11bp differences along the length (Collins *et. al.*, 1991). The homology between *L. monocytogenes* and other species of the genus *Listeria* was shown to be 99.2%, 98.8%, 98.5% and 98.4% for *L. innocua, L. welshimeri, L. seeligeri* and *L. ivanovii,* respectively (Collins *et. al.*, 1991). The stability of nucleotide differences was investigated by RAPD and sequence analysis (Czajka *et.*

al., 1993). This study showed sequence variation did occur in the V2 region of 16S rRNA between a number of *L. monocytogenes* strains and one *L. innocua* tested, but these differences did not follow any particular pattern. Therefore, analysis of the V2 region of 16S rRNA (143-220bp) by PCR primers pB/pD' would be expected to reflect this sequence instability.

Clinical isolates of both *L. monocytogenes* and *L. innocua* appeared to contain more nucleotide differences than those derived from culture collection strains to reference sequences along the experimental fragments. This may indicate divergence of *L. monocytogenes* strains on the basis of 16S rRNA sequence, but such conclusions founded on this limited data set are speculative. However, whilst no differences were observed between 23S rRNA sequence of a range of clinical and culture collection specimens of *L. monocytogenes*, study of flagellin, intra-invasive protein and listeriolysin genes has indicated evolutionary divergence which could be correlated to major serogroups (Rasmussen *et. al.*, 1995; Brosch *et. al.*, 1994).Variation was previously reported between serotypes of *L. monocytogenes*, but these were found to be unstable (Czajka *et. al.*, 1993). No correlation between serotype and 16S rRNA gene sequence could be reliably inferred from data shown in this study.

Given the limited sequence information obtained, phylogenetic trees presented in section 6.3. should be regarded as indicating only tentative relationships between species.

The non-diagnostic value of DNA sequences in the V2 region is reflected in sequence data obtained from PCR primers pB/pD'. Distance matrix analysis of experimental and reference sequences (Fig. 6.3.3.) showed experimental sequences to fall in a separate group to those containing reference sequences with the exception of *L. monocytogenes* 19113. Experimental DNA sequences, with one exception were shown to have maximum similarity to the appropriate reference sequence, which indicates the non-representative nature of sequences obtained in this region of the 16S rRNA gene.

Differences observed between 16S rRNA sequences of *L. monocytogenes* and *L. innocua* in the V9 region of the gene have been previously reported to be stable with respect to species (Collins *et. al.*, 1991; Czajka *et. al.*, 1993). Experimental sequences obtained from this region obtained with primers pF/pG' analysed by a distance matrix method showed that experimentally determined sequences were grouped with respective reference strains (Figs. 6.3.4.). This indicates that the sequences analysed were representative of their respective species and permitted tentative identification on the basis of these DNA fragment sequences. Partial nucleic acid sequences have been used with success to study components of ecosystems at kingdom to species level (Giavonnoni *et. al.*, 1988; Fuhrman *et. al.*, 1992) demonstrating the use of nucleotide sequence 'signatures' to study microbial components of an ecosystem without prior culturing. Analysis of sequences obtained from both pB/pD' and pF/pG' for four strains where this was possible showed that with the exception of *L. monocytogenes* 2073, other strains tested were grouped in close proximity to anticipated reference sequence. *L. monocytogenes* 2073 contained significantly more sequence differences to the reference *L. monocytogenes* than other *L. monocytogenes* strains examined (Fig. 6.3.2.). However, in earlier experiments, PCR fragments from this strain were shown to hybridise to oligonucleotide probes MV9P and MV9RP2 (Fig. 3.3.2.h).

Inclusion of other 16S rRNA sequences from other low G + C content bacteria showed a similar relationship to one another as shown by Collins *et. al.*, (1991) (Fig. 6.3.6.) when subjected to distance matrix analysis. In general, it should be noted that the genus *Listeria* is tightly clustered, addition of other less related outgroups to the analysis obscured the relationship between members of the genus *Listeria* (data not shown). Inclusion of the experimental data in this analysis indicated that these sequences bore closest relationship to either *L. monocytogenes* or *L. innocua* but were distinct from one another (Fig. 6.3.7.). However, the data do not conclusively separate experimental sequences were of either *L. monocytogenes* or *L. innocua* strains.

To have increased confidence in the relationship shown the phylogenetic tree structure presented in section 6.3., phylogenetic tree topology should be compared to that obtained by an alternative method of analysis, for example, maximum parsimony analysis. Maximum parsimony analysis assumes the fewest number of mutational events to explain the differences observed between the gene sequences. A common maximum parsimony method may be found in the phylogeny inference package (Felsenstein, 1982). Tree topology may be tested by bootstrap analysis which randomly re-samples the dataset to test confidence of the tree branching structure and position. In conclusion, this study indicated that *L. innocua* strain 33090 which exhibits cross reactivity with PCR primers MV2/MV9R for *L. monocytogenes* contains two tentative nucleotide differences in the primer sequences, whilst other strains, in particular *L. monocytogenes* 2073 contained a greater number of sequence differences across the fragments examined. Relationships observed between experimental and reference sequences when analysed by a distance matrix method indicated that analysis with pB/pD' did not group *L. monocytogenes* sequences separately from other species examined. Sequences pF'/pG' did however, resolve the experimental sequences, and with one exception, grouped them closest to their respective reference sequences. These results support those of Czajka *et. al.*, (1993) which demonstrated that the V2 region of the 16S rRNA gene was of dubious value in species-specific detection of *L. monocytogenes*.

CHAPTER 7.

CONCLUDING REMARKS.

Chapter 7.

Concluding Remarks.

The studies reported here have illustrated in general, the advantages and disadvantages of nucleic acid based techniques to study bacterial physiology and ecology. In particular, they have addressed the methodology of nucleic acid extraction, the relationship between nucleic acid ratios and physiology, and the feasibility of 16S rDNA amplification for the specific detection of *L. monocytogenes*.

7.1. Diagnostic PCR detection of *L. monocytogenes*.

Evaluation of PCR primers targeted at sequences of the 16S rRNA gene of *L. monocytogenes* showed cross reactivity with DNA from other members of the genus *Listeria*, in particular, *L. innocua*. Sequence analysis of the appropriate regions of the 16S rRNA gene of several *Listeria* species showed that sequence variation did occur in the area targeted by the forward PCR primer may result in cross reactivity. In terms of 16S rRNA sequence the members of the genus *Listeria* are very similar to one other (Collins *et. al.*, 1991). Comparison of nucleic acid sequence information from several *Listeria* spp. demonstrated the potential risks of mis-interpreting this information. Analysis of DNA sequences where forward PCR primer MV2 annealed showed that stable *L. monocytogenes* or *L. innocua* sequence signatures were not present. Consequently, distance matrix analysis of these sequences showed these sequences bore little similarity to the appropriate reference strain. In contrast, sequence information obtained from the area where reverse PCR primer MV9R annealed showed increased discrimination between different *Listeria* spp. Analysis of these sequences by distance matrix methods showed that with one exception, experimental sequence data grouped closest to the appropriate reference sequence. When sequence information from both fragments was combined, analysis showed that

experimental sequences generally grouped closest to the appropriate reference sequence.

Results described above showed that in this situation, *L. monocytogenes* diagnosis on the basis of 16S rRNA detection alone is unreliable. Analysis of sequence information revealed the risks involved in analysing partial 16S rRNA gene sequences from unknown organisms. The sequence examined and method of analysis used can fundamentally alter interpretation of experimental data. The degree of similarity between 16S rRNA sequences of within the genus *Listeria* has been examined previously by Czajka *et. al.*, (1991). This has led to alternatives to PCR being considered for diagnosis of *L. monocytogenes* presence. Molecular weight profiles of 5S rRNA and tRNA were unsuitable (Slade and Collins-Thompson 1991), but ligase chain reaction (Wiedmann *et. al.*, 1993) and RAPD analysis of the 16-23S ribosomal spacer region (Jensen *et. al.*, 1993; Drebot *et. al.*, 1996) have proved successful rRNA based methods. Indeed, examination of nucleic acid polymorphisms has been successfully used to trace sources of Listeriosis outbreaks (Harvey and Gilmour, 1994; Neiderhauser *et. al.*, 1994). More reliable PCR based method detection of *L. monocytogenes* may be achieved with primers directed toward pathogenicity genes, in particular *iap* genes (Bubert *et. al.*, 1992; see Table 1.6.5.1. above).

Detection of *L. monocytogenes* by molecular methods such as PCR will continue to be perceived as a confirmatory test, subservient to culture based techniques until DNA can be extracted reproducibly, and with increased sensitivity, directly from foodstuffs, or molecular probes detect nucleic acids within cells extracted from environmental samples. This study showed that DNA could be extracted from milk by published methods (Dickinson *et. al* 1995), but PCR amplification results were inconclusive. Extraction of both RNA and DNA was the goal, to permit evaluation of RNA/DNA ratios, and draw conclusions concerning activity of the organism *in-situ*. The nucleic acid extraction used in RNA/DNA ratio studies had an estimated sensitivity of $\geq 10^5$ cfu.ml^{-1}. It is unlikely that this level of

Listeria spp. persists in many foodstuffs. Promising results were obtained, but these failed to conclusively show that both DNA and RNA were extracted from milk. Future studies could exploit novel methods of cell separation from foodstuffs, for example two-phase separation (Lantz *et. al.*, 1994) and lectin coated magnetic bead concentration (Payne *et. al.*, 1992). Extraction of nucleic acids could be achieved with the use of novel procedures, such as the use of a *Listeria spp.* specific phage encoded bacteriolysin which may permit lysis and nucleic acid extraction of only *Listeria spp.* from a heterogeneous sample.

In-situ hybridisation studies were successful with a domain Bacteria specific probe, but no hybridisation was observed with a *L. monocytogenes* specific oligonucleotide probe. The area of 16S rRNA which was targeted by the species specific oligonucleotide probe has not been reported to possess inhibitory secondary structure (Amann, 1995). Detection of microorganisms *in-situ* with oligonucleotide probes is rapidly becoming a routine method of microbial analysis. The technique permits examination of selected components of microbial consortia, and if 16S rRNA directed probes are used, provide a semi-quantitative assessment of microbial activity (Amann, 1995; Amann *et. al.*, 1990a/b). Study of *L. monocytogenes* with *in-situ* hybridisation techniques will present specific problems according to the material sampled. *In-situ* hybridisation examination of microorganisms has been successfully achieved in marine bivalve tissues (Distel and Cavanaugh 1994) and detection of *L. monocytogenes* in meat tissues would present a similar challenge to the microbial ecologist. However, *in-situ* detection of the organism in other foods, for examples, cheese and processed foods, may be more problematical due to their dispersed and homogenous nature.

7.2. Assessment of Nucleic Acid Extraction.

Quantitative analysis of nucleic acid content from cell extracts requires prior

estimation of nucleic acid extraction efficiency to be meaningful. Many studies describing RNA/DNA ratios, perhaps surprisingly, pay scant regard to this consideration (Kramer and Singleton, 1992; McSweeney *et. al.*, 1993; Moyer *et. al.*, 1990). This study showed that different lysis techniques are appropriate for specific tasks. To study RNA/DNA ratios both RNA and DNA were required, preferably simultaneously. This investigation showed that enzymatic lysis was more suitable than physical disruption methods, but less nucleic acids were released from cell debris than were released by physical lysis methods. Both ultrasonication and freeze-thaw disruption were suitable for release of increased quantities of rRNA with respect to enzymatic lysis, but no DNA was detected by electrophoresis.

Quantitative oligonucleotide probing was a sensitive method of nucleic acid analysis. DNA quantitation was less sensitive than that of rRNA due to less target being present per unit mass of nucleic acid. In addition, preparation of immobilised nucleic acids required considerable care to obtain reproducible standard and experimental results. These two factors combined to limit the use of this method to assess nucleic acid ratios an environmental situation. Low cell numbers of low activity (and to consequently low rRNA content) in samples containing materials inhibitory to conventional nucleic acid extraction methods combine to preclude the methods used here from more widespread use. DNA could be assayed by quantitative PCR, but these techniques require rigorous standardisation and reproducibility is unclear, particularly in the presence of a number of DNA species (Suzuki and Giavanonni 1996). These problems may be reduced by nucleic acid extraction, particularly from foodstuffs by the methods mentioned above, or cell separation prior to nucleic acid recovery.

Fluorescent techniques used to determine nucleic acid ratios proved to be rapid, sensitive, and simple. However, analysis of whole cells and nucleic acid extracts showed some discrepancy. This was probably due in part, to both availability of nucleic acids and efficiency of extraction. Therefore, data produced by all methods used in these studies should be treated with caution before making general

conclusions concerning quantities of nucleic acids determined in different studies. Future studies could use fluorescent dyes of improved sensitivity, for example ethidium homodimer (Moyer *et. al.*, 1990), but such dyes are non-specific in nature and would consequently only be used in homogenous systems, or to study total microbial population, examination of specific constituents achieved with targeted probes.

Determination of nucleic acid ratios by oligonucleotide probing and fluorescent dye techniques predominantly showed agreement in trends observed from experiments performed. Differences in RNA/DNA ratios recorded by the different techniques were probably due to the reasons described above for fluorescent dyes, and the reliability of detecting small quantities of target DNA by oligonucleotide probing methods.

7.3. Nucleic Acid Ratios under Different Growth Conditions.

Study of nucleic acid ratios during *L. monocytogenes* growth has not been reported previously. These studies have shown that during batch growth nucleic acid ratios follow similar trends (but not quantities) to those observed for *E. coli* and a marine bacterium (Davis *et. al.*, 1986; Moyer *et. al.*, 1990, respectively). Growth in batch culture in a complex unbuffered media is rapid, and consequently RNA/DNA ratios rise quickly. However, growth results in acidification of the medium, which promptly inhibits *L. monocytogenes* growth. Nucleic acid ratios measured by different techniques all decreased at the onset of cell death. However, despite viable counts of zero, significant quantities of nucleic acids were detected. The media used to evaluate colony forming units may not be suitable for the recovery of injured cells. When *L. monocytogenes* was grown in pH controlled batch culture significant numbers of cells (10^9 cfu.ml-1) were maintained for an extended period (>2 weeks). After an initial rapid increase during exponential growth, nucleic acid ratios decreased slowly over

the remainder of the experiment. It is presumed that a basal requirement of ribosomes is required to maintain cell viability (Flärdh *et. al.*, 1992) and this phenomenon may have been observed here. Measurement of nucleic acids during these experiments probably represented an aggregate of the cell population contained within a particular sample. In addition, nucleic acid ratios observed in lag phase and death phase were of similar value. Therefore determination of RNA/DNA ratios at these points would fail to discriminate cultures with the potential for rapid growth and one of decreased vitality. Addition of fresh nutrients to cultures in stationary phase may have initiated increased RNA synthesis to support fresh growth. However, it is highly unlikely that cells contained within a foodstuff are attaining maximum growth rates and determination of nucleic acid ratios, if successful would fail to demonstrate the potential for proliferation upon transfer to a suitable environment. Production of foodstuffs by traditional methods such as drying, smoking, salting and fermenting often prevent *L. monocytogenes* proliferation and the organism is either inhibited, or localised within the food matrix. Problems are more likely to occur where non-preserved foods are stored for extended periods and increased scale and amount of processing greatly increases risk of listerial contamination throughout the food matrix.

Determination of RNA/DNA ratios with respect to growth rate using the techniques was limited by the same problems to those described. Growth, and nucleic acid ratios in starved cells using the methods described here would be better studied by continuous culture methods. Continuous culture would permit growth of a cell population that is theoretically homogenous in nature, and measurement of nucleic acid ratios would be a better reflection of nucleic acid content of cells. In addition, accurate examination could be achieved regarding the response of *L. monocytogenes* to nutrient shift-up and shift-down. Although cultures were growing at different growth rates, the sample was probably heterogeneous and any relationship between growth rate and nucleic acid ratio observed should be regarded with some caution. Of note, is the difference observed in value between fluorometric and oligonucleotide probing data which reinforces the argument concerning the importance of the

techniques used to measure nucleic acid ratios. In view of the findings on RNA/DNA ratios determined here, and those published previously the proposal that microorganisms fit a universal growth rate versus RNA/DNA ratio model (Kerkhof and Ward 1993) is regarded with scepticism. It is proposed that organisms maintain a critical number of ribosomes below which cell death occurs (Flärdh *et. al.*, 1992). Transfer to a suitable environment results in increased rRNA synthesis to permit increased cellular demands for transcription and translation. Nucleic acid ratios may increase proportionally to growth rate up to a certain level above which synthesis of further ribosomes would be impossible for the cell to accommodate. This point may be where all ribosomal RNA gene copies are being transcribed, or ribosomal proteins cannot be synthesised any faster. The exact nature of this relationship requires further investigation by comparison of nucleic acid ratios determined by the same techniques at a variety of growth rates determined in continuous culture for a number of different organisms. Multiple gene rRNA gene copies have generally been thought necessary to support a high growth rate of rRNA in rapidly dividing cells (Amikan *et. al.*, 1982; Nomura *et. al.*, 1977). This would be reflected by increased RNA/DNA ratios at identical growth rates to those shown by slower growing organisms.

Observation of nucleic acid ratios prior to, and following sublethal injury by acid and heat indicated that after decreases cell population recovered completely. Nucleic acid ratios have not been previously examined in such a situation before. Injury of *L. monocytogenes* cultures by acid in minimal media showed that choice of lethal agent is of the utmost importance when studying cell death and injury in food processing environments. Addition of concentrated HCl showed minimal effect on both mid-exponential and stationary phase cultures of *L. monocytogenes* in minimal media. Heat injury of *L. monocytogenes* cells showed that recovery was rapid in cells grown at an increased temperature prior to heat shock, and that recovery only occurred in a complex medium. The principle problem with these experiments was that no differentiation was made between injured and healthy cells. Future studies should examine the recovery of injured cells. This may be achieved by use of plating

media that contains NaCl, to which heat, or acid injured *L. monocytogenes* cells are sensitive (Bunduki *et. al.*, 1995). Acid injury with HCl was poor and future studies should employ an organic acid, for example acetic acid (Ita and Hurkins 1991). However, recovery of injured cells as a population is possibly a better reflection of bacterial survival and growth in a food processing situation due to the heterogeneous structure of many foodstuffs and presence of biofilms in the food processing environment. Recovery from heat injury was probably successful only in complex media because injury caused loss of membrane competence, consequent loss of ions and ultimately ribosomal dissociation. Ribosomal sub-units were possibly degraded and cell death occurred. Recovery in complex media was observed, probably due to presence of osmo-protectants in complex media, such as betaine and carnitine that prevented ribosomal dissociation. In addition, ample supplies of complex biochemicals were available for cellular repair which probably had the effect of reducing maintenance energy demand, further aiding cellular recovery. Greater injury was observed in cell samples grown at a lower temperature prior to heat shock. This may have been due to decreased membrane viscosity.

For molecular methods to replace culture based techniques as the principle methods of detection and enumeration, the problems of sensitivity and inhibition must be addressed. Promising novel techniques for separation of cells from foodstuffs prior to nucleic acid extraction and bulk nucleic acid extraction have been reported previously. A specific *in-situ* method would negate problems which can occur due to nucleic acid extraction efficiency. Use of ribosomal RNA targeted probes would permit the analysis of cellular activity in a semi-quantitative manner. However, it is unlikely that rapid growth of *L. monocytogenes* occurs in many food environments. Therefore confirmation of the presence may be desired using a PCR assay. Unfortunately, the detection of *L. monocytogenes* is not indicative of activity. In turn, the activity of the organism in a foodstuff is not indicative of its pathogenic potential if transferred to a suitable environment.

7.4. Conclusions.

- Use of 16S rRNA sequences to detect a single species in a genus with closely related sequences may be problematical.

- Nucleic acid extraction methods are highly variable in efficiency.

- Physical methods of lysis yield increased amounts of nucleic acids, susceptible to degradation with respect to enzymatic methods.

- An assessment of nucleic acid extraction efficiency is required to determine quantity and quality of nucleic acid extracts prior to the determination of truly quantitative nucleic acid ratios.

- RNA/DNA ratios may be determined successfully by fluorometric and oligonucleotide probing methods, although conclusions should not be drawn from evaluation by any single technique.

- Nucleic acid ratios follow similar patterns to those reported for other microorganisms in batch growth, although dynamics and values may be specific to *L. monocytogenes* growth.

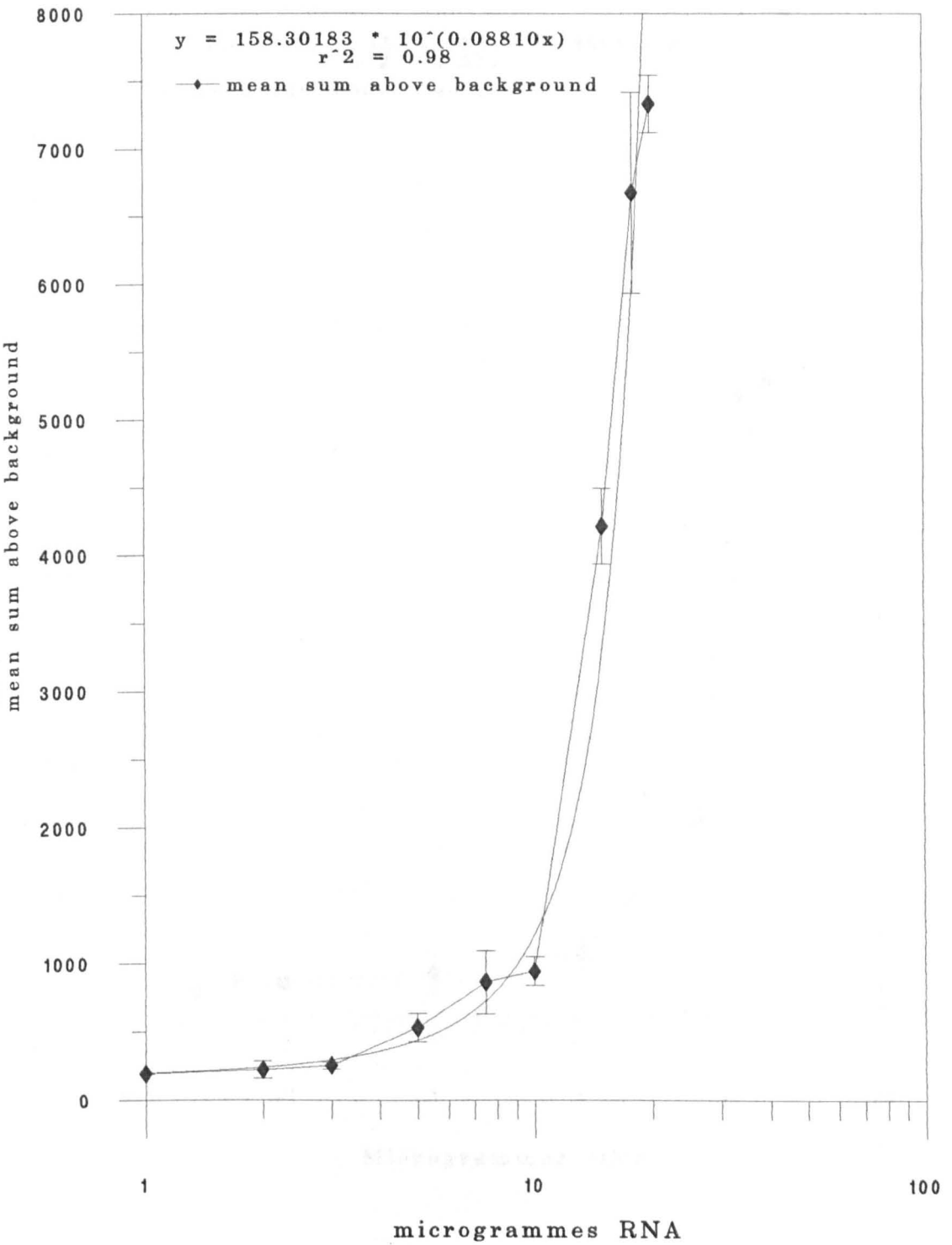
- It is probable that nucleic acid ratios are proportional over a variety of growth rates, but a residual RNA/DNA ratio is observed during prolonged stationary phase.

- Changes in RNA/DNA ratios are greater in cells grown at lower temperatures than those grown near to the thermal shocking temperature. The degree of nucleic acid ratio change is reflected in the changes in colony forming units after heat injury.

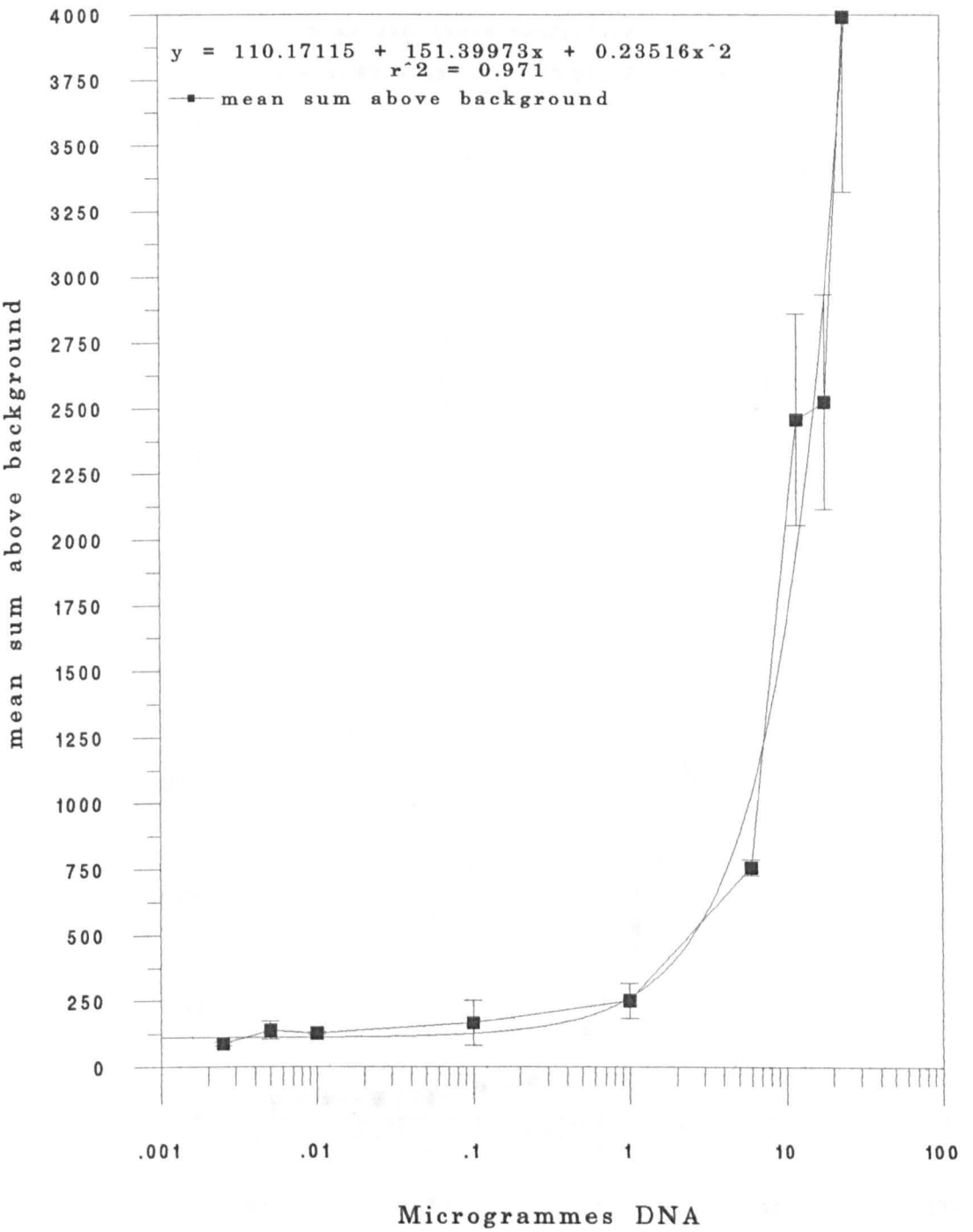
- Efficient, and rapid recovery from thermal injury may be achieved in a complex medium.

APPENDICES

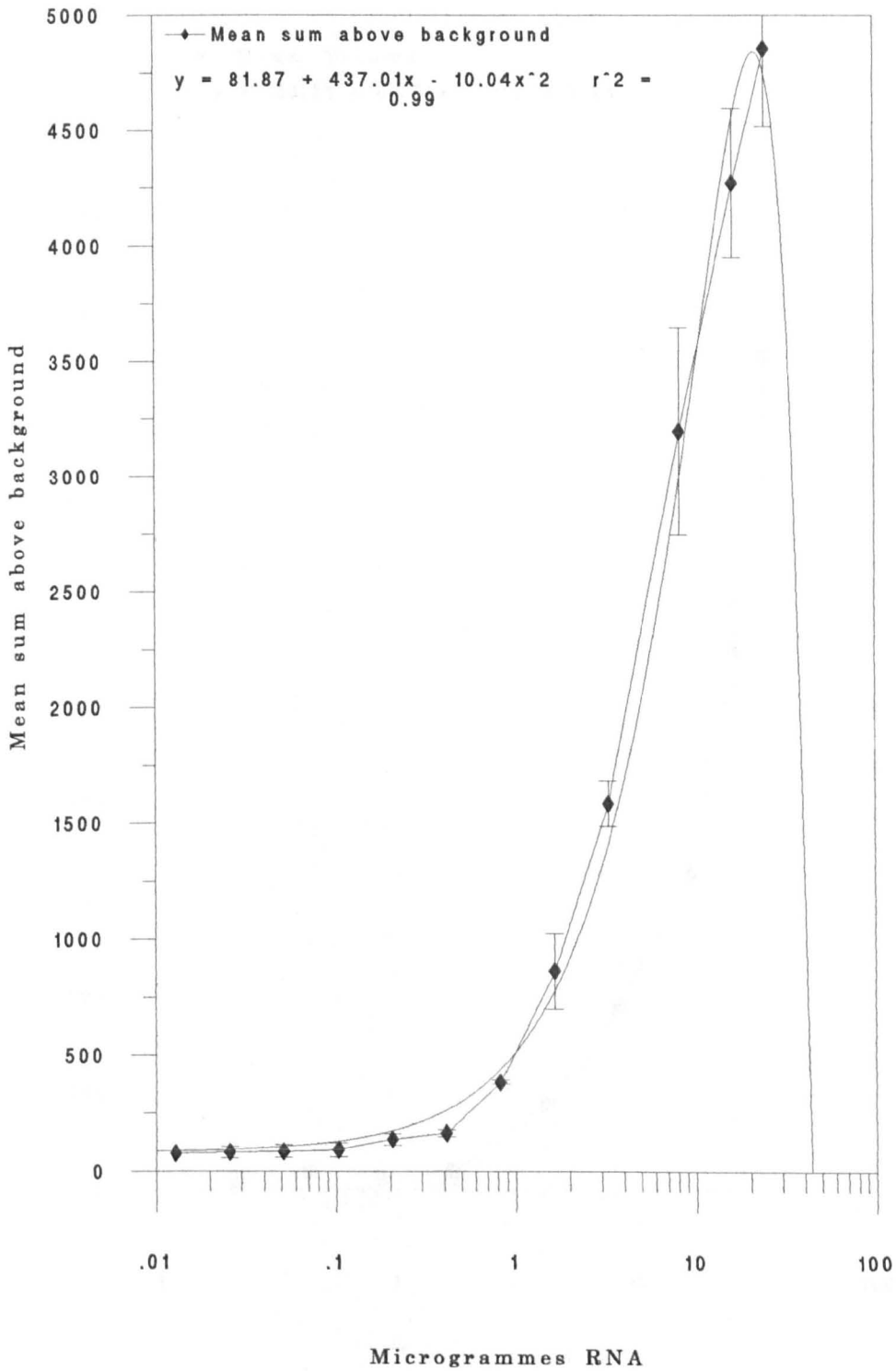
Appendix 5.6.1. Standard curve of RNA concentration determined by scanning densitometry for pH controlled batch culture of L.monocytogenes (Fig. 5.6.2.5.).



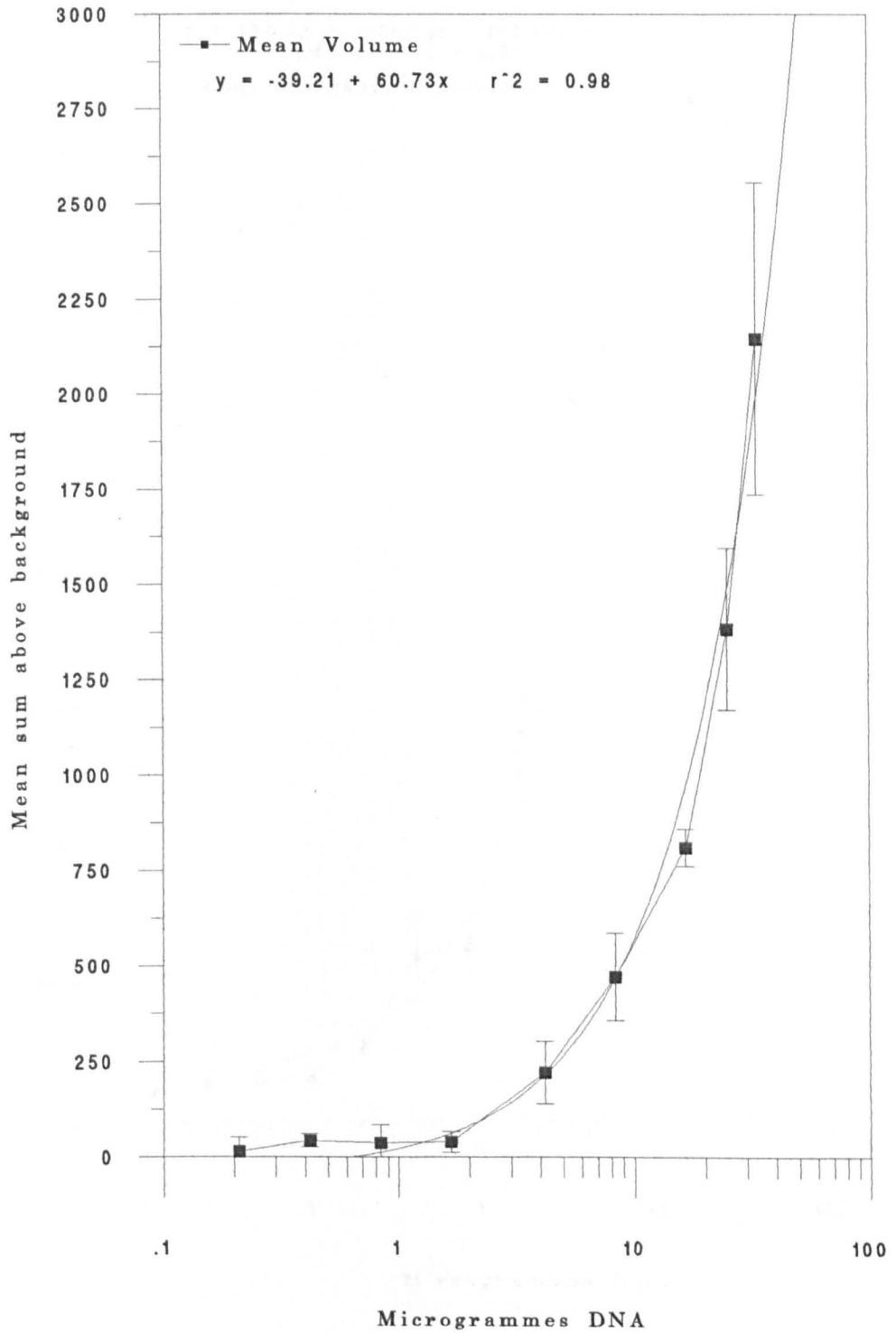
Appendix 5.6.2 Standard curve of DNA concentration determined by scanning densitometry for pH controlled batch growth of L. monocytogenes (Fig. 5.6.2.5.).



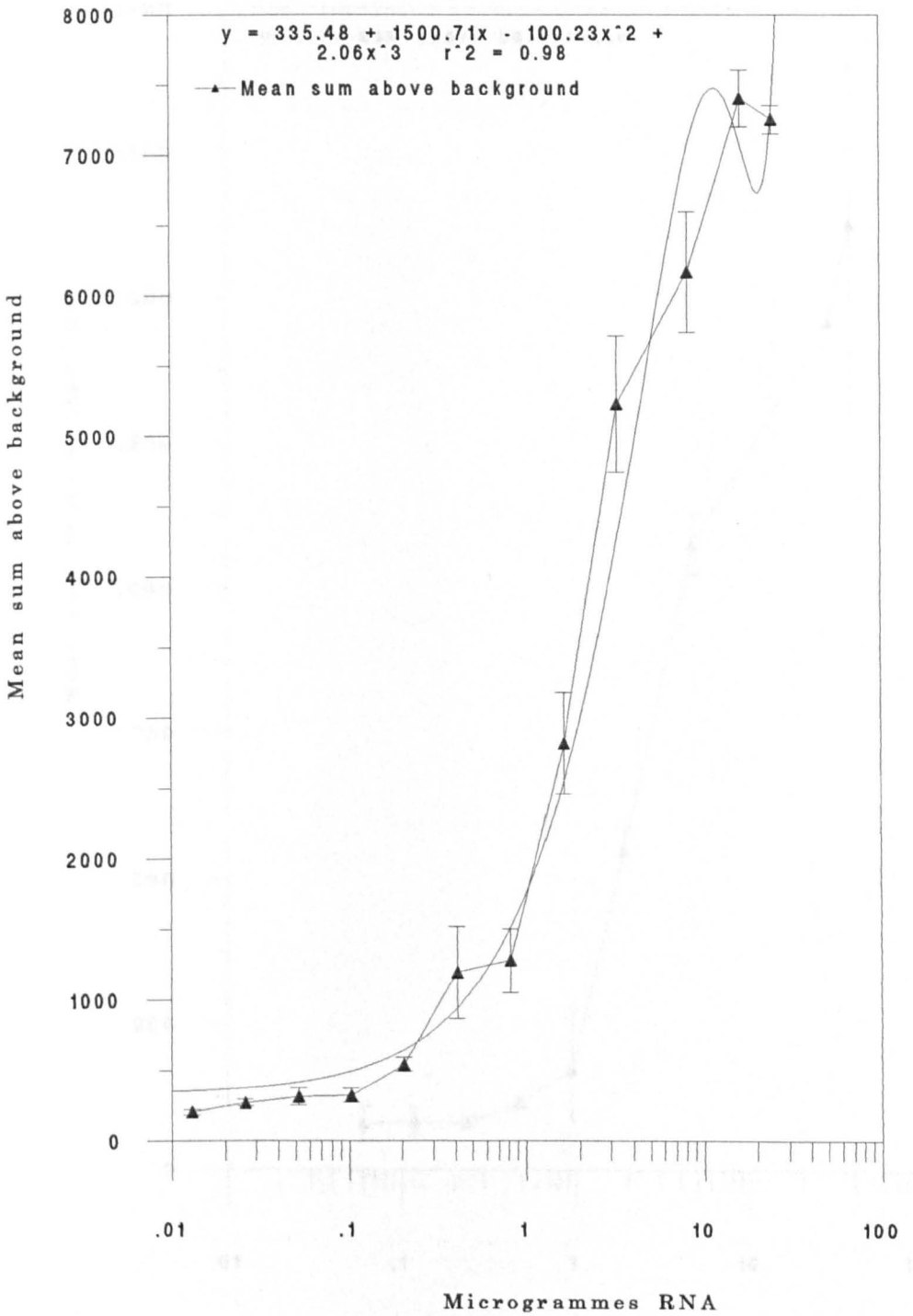
Appendix 5.7.1. Standard curve of RNA concentration determined by scanning densitometry for growth rate experiment (Fig. 5.7.4).



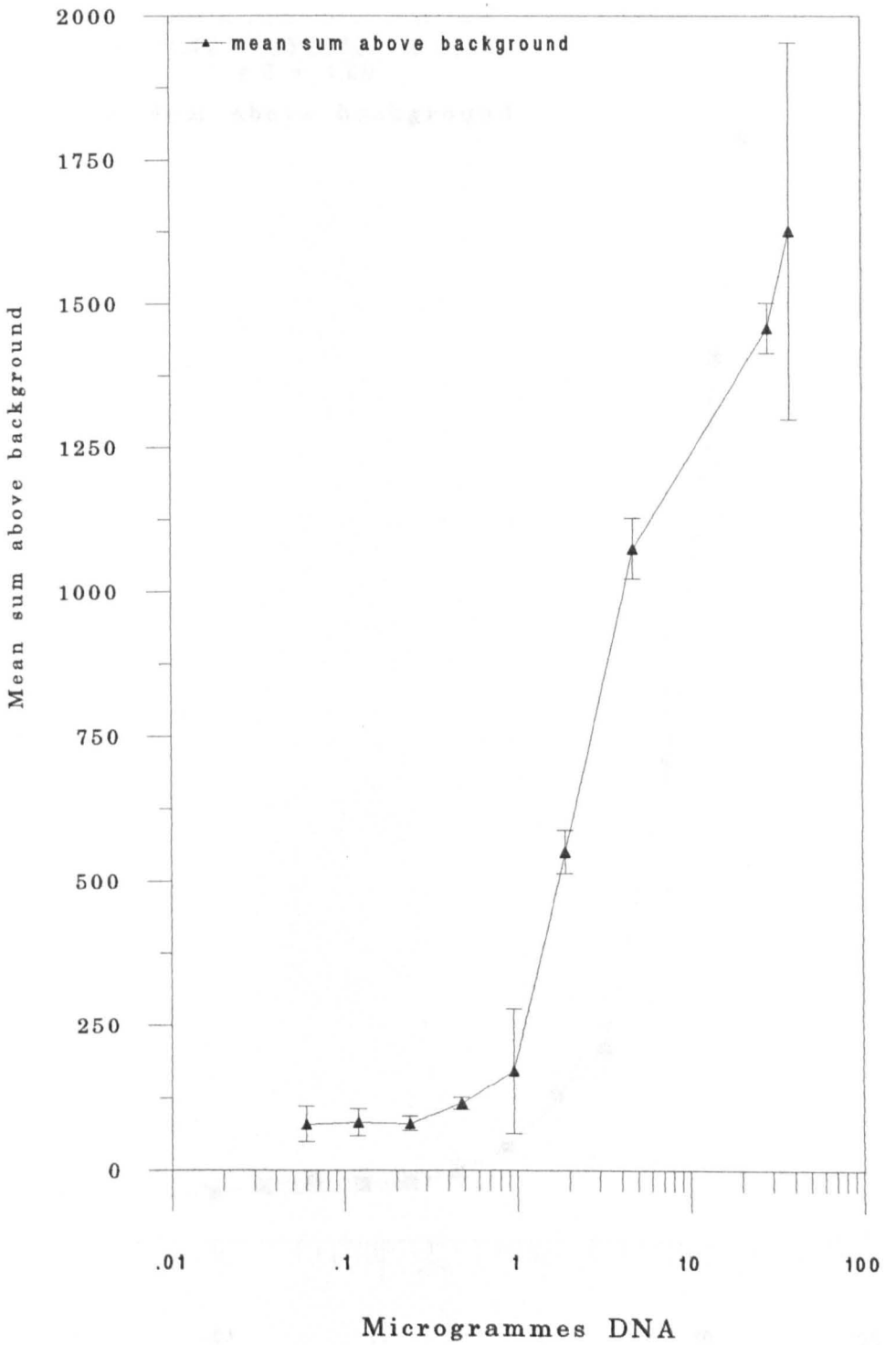
Appendix 5.7.2. Standard curve of DNA concentration determined by scanning densitometry for growth rate experiment (Fig. 5.7.4.).



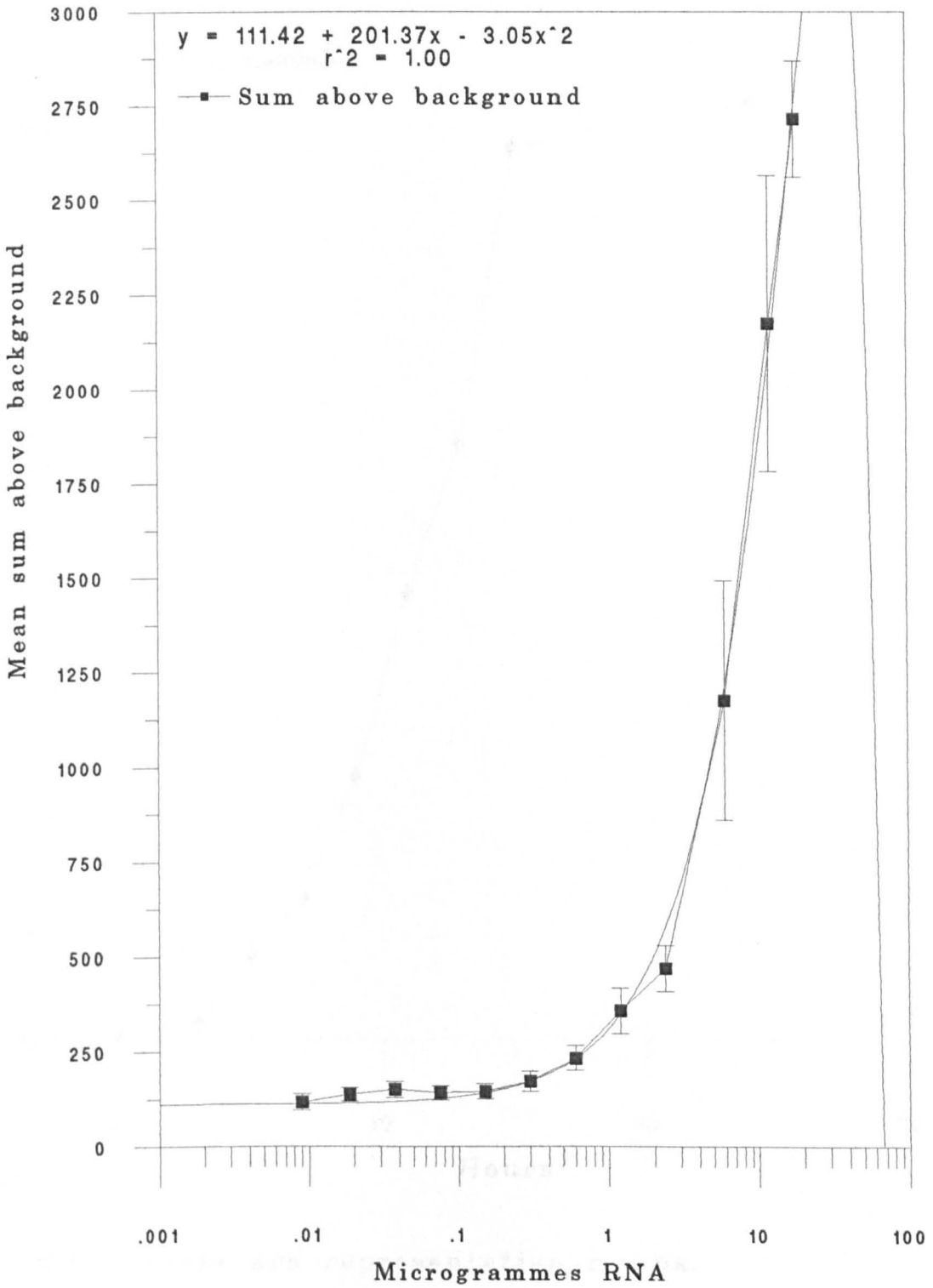
Appendix 5.8.1. Standard curve of RNA concentration against pixel intensity for acid exposure experiments (Fig. 5.8.2.).



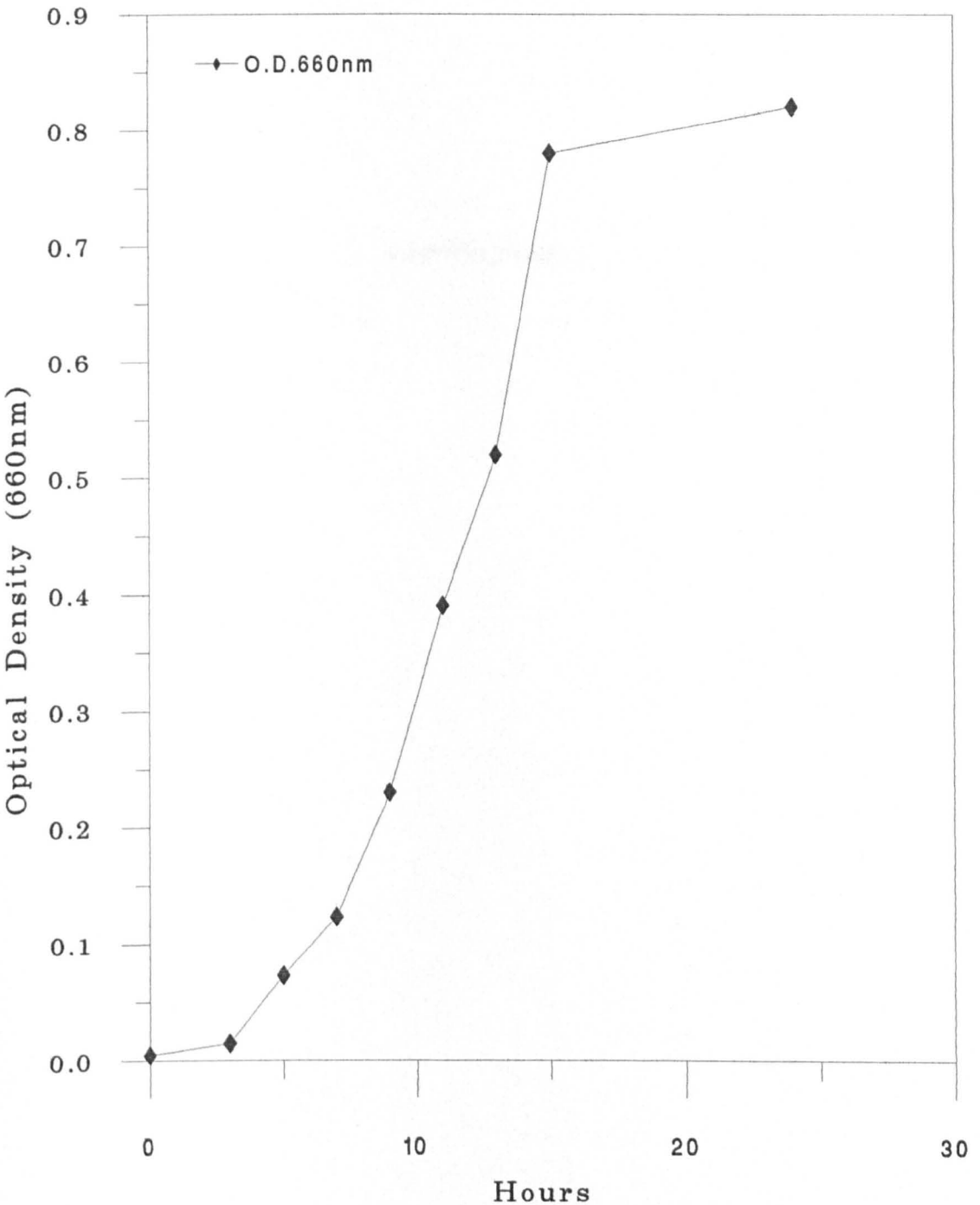
Appendix 5.8.2. Standard curve of DNA concentration against mean pixel sum above background for acid exposure experiments (Figs. 5.8.2. & 5.8.4.).



Appendix 5.8.3. RNA concentration against mean pixel sum above background for oligonucleotide probing analysis of RNA extracts from acid exposure experiment 2 (Fig. 5.8.4.).



Appendix 5.8.4. L. monocytogenes growth in Trivett and Meyer minimal media measured by optical density at 660nm (Section 5.10.3.).



N.B. Points are representative means.

REFERENCES.

REFERENCES.

Amann, R.I.; Binder, B.J.; Olson, R.J.; Chisholm, S.W.; Devereux, R. and Stahl, D.A. (1990a).
Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analysing mixed microbial populations.
Applied and Environmental Microbiology. **56**:1919-1925.

Amann, R.I.; Krumholz, L. and Stahl, D.A. (1990b),
Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic and environmental studies in microbiology.
Journal of Bacteriology. **172**:762-770.

Amann, R.I.;Ludwig, W. and Schleifer, K.-H. (1995).
Phylogenetic Identification and In Situ Detection of Individual Microbial Cells without Cultivation.
Microbiological Reviews. **59:** 143-169.

Amezaga, M.-R.; Davidson, I.; McLaggan, D.; Verheul A.; Abee, T. and Booth, I.R. (1995).
The role of peptide metabolism in the growth of *Listeria monocytogenes* ATCC 23074 at high growth osmolarity.
Microbiology. **141**:41-49.

Amikam, D.; Razin, S. and Glaser, G. (1982).
Ribosomal genes in Mycoplasma.
Nucleic Acids Research. **10**:4215-4222.

Anderson, W.A.; Hedges, N.D.; Jones, M.V. and Cole, M.B. (1991).
Thermal inactivation of *L. monocytogenes* studied by differential scanning calorimetry.
Journal of General Microbiology. **137**:1419-1424.

Anderson, M.L.M. and B.D. Young. (1985).
Quantitative filter hybridisation, p.73-110.
In: B.D.James and S.J. Higgins (eds.), Nucleic acid Hybridisation. Oxford University Press, Oxford.

Andrews, A.T. (1983).
Proteinases in normal bovine milk and their action on caesins.
Journal of Dairy Research. **50:**45-55.

Barry, T., Geary, S.; Hannify, S.; MacGearaillt, C.; Shalloo, M.; Heery, D.; Gammon, F. and Powell, R. (1993).
Rapid Minipreparations of total RNA from Bacteria.
Nucleic Acids Research. **20:**4940.

Baxter, F.; Wright, F.; Chalmers, R.M.; Low, J.C. and Donachie, W. (1993).
Characterisation by multilocus enzyme electrophoresis of *Listeria monocytogenes* isolates involved in ovine Listeriosis outbreaks in Scotland from1989 to 1991.
Applied and Environmental Microbiology. **59**:3126-3129.

Belitz,H.D. and Grosch, W. (1986).
Food Chemistry, Springer Verlag, Berlin, Germany.

Beresford, T. and Condon, S. (1991).
Physiological and genetic regulation of rRNA synthesis in *Lactococcus.*
Journal of General Microbiology. **139:**2009-2017.

Berry, E.D.; Liewen, M.B.; Mandigo, R. and Hutkins, R.W. (1990).
Inhibition of *Listeria monocytogenes* by bacteriocin producing *Pediococcus* during the manufacture of fermented semi-dry sausage.
Journal of Food Protection. **53**:681-686.

Bessesen, M.T.; Luo, Q.; Rotbart, H.A.; Blaser, M. and Ellison, R.T. (1990).
Detection of *Listeria monocytogenes* by using the Polymerase Chain Reaction.
Applied and Environmental Microbiology. **56**:2930-2932.

Beuchat, L.R. and Brackett, R.E. (1990)
Inhibitory effect of raw carrots on *Listeria monocytogenes.*
Applied and Environmental Microbiology. **56**:1734-1742.

Beuchat, L.R. and Worthington, R.E. (1975).
Relationships between heat resistance and phospholipid fatty acid composition of *Vibrio parahaemolyticus.*

Applied and Environmental Microbiology. **31**:389-394.

Beumer, R.R.; Te Giffel, M.C.; Cox, L.J.; Rombouts, F.M. and Abee, T. (1994).
Effect of Exogenous Proline, Betaine and Carnitine on Growth of *Listeria monocytogenes* in a minimal medium.
Applied and Environmental Microbiology. **60**:1359-1363.

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

Black, S.F.; Gray, D.I.; Fenlon, D.R. and Kroll, R.G. (1995).
Rapid RAPD analyis for distinguishing *Listeria* species and *Listeria monocytogenes* serotypes using a capillary air thermal cycler.
Letters in Applied Microbiology. **20**:188-190.

Blackburn, C. de W. (1993).
Rapid and Alternative methods for the detection of salmonellas in foods.
Journal of Applied Bacteriology. **75**:199-214.

Blais, B.W. and Phillippe, L.M. (1993).
A Simple RNA Probe System for Analysis of *Listeria monocytogenes* Polymerase Chain Reaction Products.
Applied and Environmental Microbiology. **59**:2795-2800.

Blumberg, D.B. (1987).
Creating a ribonuclease free environment.
Methods in Enzymology. **15**:20-24

Bobitt, J.A. and Betts, R.P. (1992).
The removal of bacteria from solutions by membrane filtration.
Journal of Microbiological Methods. **16**:215-220.

Boddy, L. and Wimpenny, J.W.T. (1992).
Ecological Concepts in Food Microbiology.
Society for Applied Bacteriology Symposium Series Number **21**:23S-38S.

Boye, M.; Ahl, T. and Molin, S. (1995).
Application of a Strain-Specific rRNA Oligonucleotide Probe Targeting *Pseudomonas fluorescens* Ag1 in a Mesocosm Study of Bacterial Release into the Environment.
Applied and Environmental Microbiology. **61**:1384-1390.

Braun-Howland, E.B.; S.A. Danielson and S.A. Nierzwicki-Bauer. (1992).
Development of a rapid method for detecting bacterial celss *in situ* using 16S rRNA-targeted probes.
Biotechniques. **13**:928-933.

Bremer H. and P. Dennis. (1987).
Modulation of Chemical Composition and Other Parameters of the Cell by Growth Rate. *In: Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology. Eds.: F. C. Neidhart; J. L. Ingraham; K. B. Low; B. Magasanik; M. Shaechter and H.E. Umbarger. Published: Washington D.C. 1987.

Brosch, R.; Chen, J. and Luchansky, J.B. (1994).
Pulsed-Field Fingerprinting of Listeriae: Identification of Genomic Divisions for *Listeria monocytogenes* and Their Correlation with Serovar.
Applied and Environmental Microbiology. **60**:2584-2592.

Bsat, N. and Batt, C.A. (1993).
A combined modified recerse dot-blot and nested PCR assay for the specific non-radioactive detection of *Listeria monocytogenes.*
Molecular and Cellular Probes. 7:199-207.

Bubert, A.; Kohler, S. and Goebel, W. (1992).
The homologous and heterologous regions within the iap gene allow genus- and species-specific identification of *Listeria* spp. by polymerase chain reaction.
Applied and Environmental Microbiology. **58**:2625-2632.

Buchanan, R.L. and Phillips. (1990).
Response surface model for prediicting the effects of temperature, pH, sodium chloride content, sodium nitrite concentration and atmosphere on the growth of *Listeria monocytogenes.*
Journal of Food Protection. **53**:370-376.

Bunduki, M.M.-C.; Flanders, K.J. and Donnelly, C.W. (1995).
Metabolic and Structural Sites of Damage in Heat- and Sanitizer-Injured Populations of *Listeria monocytogenes*.
Journal of Food Protection. **58**:410-415.

Bunning, V.K.; Donnelly, C.W.; Peeler, J.T.; Briggs, E.H.; Bradshaw, J.G.; Crawford R.G.; Beliveau, C.M. and Tierney , J.T. (1988).
Thermal inactivation of *Listeria monocytogenes* within bovine milk phagocytes.
Applied and Environmental Microbiology. **54**:364-370.

Busch S.V. and Donnelly, C.W. (1992).
Development of a Repair-Enrichment Broth for Resuscitation of Heat-Injured *Listeria monocytogenes* and *Listeria innocua.*
Applied and Environmental Microbiology. **58**:14-20.

Caldwell, D.E.; Korber, D.R. and Lawrence, J.R. (1992).
Imaging of bacterial cells by fluorescence exclusion using scanning confocal laser microscopy.
Journal of Microbiological Methods. **15**:249-261.

Cano, R.J.; Norton, D.M; Inzunza, A.E.; Sanchez, J.G. and Oste, C. (1995).
Polymerase Chain Reaction Assay Coupled with Fluorescence Detection on Microwell Plates for *Listeria monocytogenes* in Foods.
Journal of Food Protection. **58**:614-620.

Carriere, C.; Allardet-Servent, A.; Bourg, G.; Audirier, A. and Ramuz, M. (1991).
DNA polymorphism in strains of *Listeria monocytogenes.*
Journal of Clinical Microbiology. **29**:1351-1355.

Chomczynski, P. (1992).
Solubilisation in formamide protects RNA from degradation.
Nucleic Acids Research. **20**:3791-3792.

Chou, Q.; Russell, M.; Birch, D.E.; Raymond, J. and Bloch, W. (1992).
Prevention of pre-PCR mis-priming and primer dimerisation improves low-copy-number amplifications.
Nucleic Acids Research. **20**:1717-1723.

Christie R.; Atkins N.E. and Munch Petersen. (1944).
A note on a lytic phenomenon shown by group B streptococci.
Australian Journal of Experimental and Biological Medical Science. **22**:197-200.

Cooray, K.J.; Nishibori, T.; Xiong, H.; Matsuyama, T.; Fujita, M. and Mitsuyama, M. (1994).
Detection of Multiple Virulence-Assoscated Genes of *Listeria monocytogenes* by PCR in Artificially Contaminated Milk Samples.
Applied and Environmental Microbiology. **60**:3023-3026.

Cowart, R.E. and Foster, B.G. (1985).
Differential effects of iron on the growth of *Listeria monocytogenes*: minimum requirements and mechanism of aquisition.
Journal of Infectious Diseases. **151**:721-730.

Czajaka, J.; Bsat, N.; Piani, M.; Russ, W.; Sultana, K.; Wiedmann, M.; Whitaker, R. and Batt, C.A. (1993).
Differentiation of *Listeria monocytogenes* and *Listeria innocua* by 16S rRNA Genes and Intraspecies Discrimination of *Listeria monocytogenes* Strains by Random Amplified Polymorphic DNA Polymorphisms.
Applied and Environmental Microbiology. **59**:304-308.

Datta, A.R.; Wentz, B.A. and Hill, W.E. (1987).
Detection of hemolytic *Listeria monocytogenes* by using DNA colony hybridisation.
Applied and Environmental Microbiology. **53**:2256-2259.

Datta, A.R. and Kothary, M.H. (1993).
Effects of Glucose, Growth Temperature, and pH on Listerioloysin O Production in *Listeria monocytogenes.*
Applied and Environmental Microbiology. **59**:3495-3497.

Davis, B.D.; Luger, S.M. and Tai, P.C. (1986).
Role of Ribosome Degredation in the Death of Starved *Escherichia coli* Cells
Journal of Bacteriology. **166**:439-445.

DeLong, E.F.; G.S. Whickham and N.R. Pace. (1989).
Phylogenetic stains: ribosomal RNA-based probes for the identification of single microbial cells.
Science. **243:**1369-1363.

DeSoete, G. (1983).
A least squares algorithm for fitting additive trees to proximity data.
Psychometrika. **48**:621-626.

Deneer, H.G. and Boychuk, I. (1991).
Species-specific detection of *Listeria monocytogenes* by DNA amplification.
Applied and Environmental Microbiology. **57**: 606-609.

Deneer, H.G.; Healey, V. and Boychuk, I. (1995).
Reduction of exogenous ferric iron by a surface associated ferric reductase of *Listeria spp.*
Microbiology **141**:1985-1992.

Devereux, Haeberli and Smithies (1984).
A Comprehensive Set of Sequence Analysis Progrmas for VAX.
Nucleic Acids Research. **12**:387-395.

Dickinson, J., R. Kroll, K. Grant. (1995).
The direct application of the polymerase chain reaction to DNA extracted from foods.
Letters in Applied Microbiology **20:**212-216.

Dickson, J.S. (1990).
Survival and growth of *Listeria monocytogenes* on beef tissue surfaes as affected by simulated processing conditions
Journal of Food Safety. **10**:165-174.

Dillis, S.S.; Aperson, A.; Schmidt, M.R. and Saier, H.M. (1980).
Carbohydrate transport in bacteria.
Microbiological Reviews **44**:385.

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

Distel , D.L.; and Cavanaugh, C.M. (1994).
Independant Phylogenetic Origins of Methanotrophic and Chemoautotrophic Bacterial Endosymbioses in Marine Bivalves.
Journal of Bacteriology. **176**: 1932-1938.

Don, R.H.; Cox, P.T.; Wainwright, B.J.; Baker, K. and Mattick, J.S. (1992).
'Touchdown' PCR to circumvent spurious priming during gene amplification.
Nucleic Acids Research. **19:**4008.

Donnelly, C.W. and Baignet, G.J. (1986).
Method for Flow cytometric detection of *Listeria monocytogenes* in milk.
Applied and Environmental Microbiobiology. **52**:689-695.

Dortch, Q.; Roberts, T.; Clayton, J. and Ahmed, S. (1983).
RNA/DNA ratios and DNA concentrations as indicators of growth rate and biomass in planktonic marine organisms.
Marine Ecology Progress Series **13:**61-71.

Doyle, M.P. (1988).
Effect of environmental and processing conditions on *Listeria monocytogenes.*
Food Technology. **42**:169-171.

Doyle, M.P.; Glass, K.A.; Beery, J.T.; Garcia, G.A.; Pollard, D.J.; Schultz, R.D. (1987).
Survival of *Listeria monocytogenes* in milk during high-temperature short-time pasteurisation.
Applied and Environmental Microbiology **53**:1433-1438.

Drebot, M.; Neal, S.; Schlech, W. and Rozee, K. (1996).
Differentiation of *Listeria* isolates by PCR amplicon profiling and sequence analysis of 16-23S rRNA internal transcribed spacer loci.
Journal of Applied Bacteriology. **80**:174-178.

Dunning, A.M.; Talmud, P. and Humphries, S.E. (1988).
Errors in the polymerase chain reaction.
Nucleic Acids Research. **16**:1033-1039.

Earnshaw, R.G. and Banks, J.G. (1989).
A not on the inhibition of *Listeria monocytogenes* NCTC 11994 in milk by an activated lactoperoxidase system.
Letters in Apllied Microbiology. **8**:203-205.

Easter, M.C. and Gibson, D.M. (1985).
Rapid and automated detection of salmonella by electrical measurements.
Journal of Hygiene. **94**:245-262.

Edwards, U.; Rogall, T.; Blöcker, H.; Emde, M. and Böttger, E.C. (1989).
Isolation and direct complete nucleotide sequence of entire genes. Characterisation of a gene coding for 16S ribosomal RNA.
Nucleic Acids Research. **17**:7843-7853.

Eisen, J.A.; Smith, S.W. and Cavanaugh, C.M. (1992).
Phylogenetic Relationships of Chemautotrophic Bacteria Symbionts of *Solemya velum* Say (Mollusca: Bivalvia) Determined by 16S rRNA Gene Sequence Analysis.
Journal of Bacteriology. **174**:3416-3421.

Embley, T.M. (1991).
The linear PCR reaction: a simple and robust method for sequencing amplified rRNA genes.
Letters in Apllied Microbiology. **8**:203-205.

Fach, P.; Hauser, D.; Gulliou, J.P. and Popoff. (1993).
Polymerase chain reaction for the rapid identification of *Clostridium botulinum* type A strains and detection in food samples.
Journal of Applied Bacteriology. **75**:234-239.

Farber, J.M. and Addison, C.J. (1994).
RAPD typing for distinguishing species and strains in the genus *Listeria*.
Journal of Applied Bacteriology. **77**:242-250.

Farber, J.M., Coates, F. and Daley, E. (1992).
Minimum water activity requirements for the growth of *Listeria monocytogenes*.
Letters in Applied Microbiology. **13**:171-174.

Farber, J.M. and Peterkin, P.I. (1991).
Listeria monocytogenes, a Food-Borne Pathogen
Microbiological Reviews. **55**:476-511.

Felsenstein, J. (1982).
Numerical Methods for Inferring Evolutionary Trees.
The Quarterly Review of Biology. **57**:379-409.

Felsenstein, J. (1989).
PHYLIP-pylogeny inference package (version 3.2.).
Cladistics: **5**:164-166.

Fenlon, D.R.; Stewart, T. and Donachie, W. (1995).
The incidence, numbers and types of *Listeria monocytogenes* isolated from farm bulk tank milks.
Letters in Applied Microbiology. **20**:57-60.

Ferron, P. and Michard, J. (1993).
Distribution of *Listeria spp.* in confectioners pastries from Western France: comparison of enrichment methods.
International Journal of Food Mirobiology. **18**:289-303.

Fitter, S.; Heuzenroder, M. and Thomas, C.J. (1992).
A combined PCR and selective enrichment method for rapid detection of *Listeria monocytogenes.*
Journal of Applied Bacteriology. **73**:53-59.

Flardh, K.; Cohen, P.S. and Kjelleberg, S. (1992).
Ribosomes Exist in Large Excess over the Apparent Demand for Protein Synthesis during Carbon Starvation in Marine *Vibrio* sp. Strain CCUG 15956.
Journal of Applied Bacteriology. **174**:6780-6788.

Fluit, A.C.; Torensma, R.; Visser, M.J.C.; Aarsman, C.J.M.; Poppelier, M.J.J.G.; Keller, B.H.I.; Klapwijk, P.and Verhoef., J. (1993).
Detection of *Listeria monocytogenes* in cheese with the magnetic immuno-polymerase chain reaction assay.
Applied and Environmental Microbiology. **59**:1289-1293.

Friedmann, M.E. and Roessler, W.G. (1962).
Growth of *Listeria monocytogenes* in defined media.
Journal of Bacteriology. **82**:528-537.

Furhman, J.A.; McCallum, K. and Davis, A.A. (1992).
Novel major archaebacterial group from marine plankton.
Nature. **356**:148-149.

Gausing, K. (1977).
Regulation of Ribosome Producation in *Escherichia coli*: Synthesis and Stability of Ribosomal RNA at Different Growth Rates.
Journal of Molecular Biology. **115**:335-354.

George, S.M. and Lund, B.M. (1992).
The effect of culture medium and aeration on growth of *Listeria monocytogenes* at pH 4.5.
Letters in Applied Microbiology. **15**:49-52.

Georgellis, D.; Ardvison, S. and von Gabain,A. (1992).
Decay of *ompA* mRNA and
processing of 9S rRNA are immediately affected by shifts in growth rate, but in opposite manners.
Journal of Bacteriology. **174**:5382-5392.

Giavanonni, S.J.; DeLong, E.F.; Olsen, G.J. and Pace, N.R. (1988).
Phylogenetic Group-Specific Oligodeoxynucleotide Probes for Identification of Single Microbial Cells.
Journal of Bacteriology. **170**:720-726.

Gibson, D.M. (1987).
Some modifications to the media for rapid automated detection of salmonellas by conductance measurements.
Journal of Applied Bacteriology. **63**:299-304.

Gilbert, R.J.; Miller, K.L. and Roberts, D. (1989).
Listeria monocytogenes and chilled foods.
Lancet **i**:383-384.

Gilliand, G.; Perrin, S.; Blanchard, K. and Bunn, H.F. (1990).
Analysis of cytokine mRNA and DNA: Detection and quantitation by competetive polymerase chain reaction.
Proceedings of the National Academy of Sciences USA. **87**:2725-2729

Giraffa, G.; Carminati, D. and Tarelli, G.T. (1995).
Inhibition of *Listeria innocua* in milk by bacteriocin-producing *Enterococcus faecium* 7C5.
Journal of Food Protection. **58**:621-623.

Grant, K.A.; Dickinson, J.H.; Payne, M.J.; Campbell, S.; Collins, M.D. and Kroll, R.G. (1993).
Use of the polymerase chain reaction and 16S rRNA sequences for the rapid detection of *Brochothrix spp.* in foods.
Journal of Applied Bacteriology. **74**:260-267.

Gray, D.I. and Kroll, R.G. (1995).
Polymerase chain reaction amplification of the fla A gene for the rapid identifiaction of *Listeria spp.*
Letters in Applied Microbiology. **20**:65-68.

Gray, M.L.; Stafseth, H.J.; Thorp, H.J.; Sholl, L.B.; and Riley, W.F. (1948).
A new technique for isolating listerellae from the bovine brain.
Journal of Bacteriology. **55**:471-476.

Hahn, D.; Amann, R.I. and Zeyet, J. (1993).
Whole-Cell Hybridisation of *Frankia* Strains with Fluorecence- or Dioxygenin-Labelled, 16S rRNA-Targeted Oligonucleotide Probes.
Applied and Environmental Microbiology. **59**:1709-1716.

Hapke, B. and Noll, H. (1976).
Structural Dynamics of Bacterial Ribosomes: IV; Classification of Ribosomes by Subunit Interaction.
Journal of Molecular Biology. **105**:97-109.

Harvey J. and A. Gilmour. (1994).
Application of Multilocus Enzyme Electrophoresis and Restriction Fragment Length Polymorphism Analysis to the Typing of *Listeria monocytogenes* Strains Isolated from Raw Milk, Nondairy Foods, Clinical and Vetinary Sources.
Applied and Environmental Microbiology. **60:**1547-1533.

Herbert, D. P.J.Phipps and R.E. Strange. (1971).
Chemical Analysis of Microbial Cells.
Methods in Microbiology **5B:**282-328.

Herman, L.M.F.; De Block, J.H.G.E. and Moermans, R.J.B. (1995).
Direct Detection of *Listeria monocytogenes* in 25 millilters of Raw Milk by a Two-Step PCR with Nested Primers.
Applied and Environmental Microbiology. **61**:817-819.

Higuchi, R.; Fockler, C.; Dollinger, G. and Watson, R. (1993).
Kinetic PCR Analysis: Real-time Monitoring of DNA Amplification Products.
Bio/technology. **11**:1026-1030.

Hiorns, W.D. (1991).
16S rRNA-Targeted Probes for the Detection of Autotrophic-Ammonia Oxidising Bacteria.
Ph.D. Thesis; University of Liverpool.

Hof, H. and Rocourt, J. (1992).
Is any strain of *Listeria monocytogenes* detected in food a health risk?
International Journal of Food Microbiology. **16**: 173-181.

Hodson, R.E.; Dustman, W.A.; Garg, R.P. and Moran, M.A. (1995).
In Situ PCR for Visualisation of Microscale Distribution of Specific Genes and Gene Products in Prokaryotic Communities.
Applied and Environmental Microbiology. **61**:4074-4082.

Ita, P.S. and Hurkins, R.W. (1991).
Intracellular pH and Survival of *Listeria monocytogenes* Scott A in Tryptic Soy Broth Containing Acetic, Lactic, Citric and Hydochloric Acids.
Journal of Food Protection. **54**:15-19.

Jensen, K.F. and Pedersen, S. (1990).
Metabolic growth rate in *Eschericia coli* may be a consequence of subsaturation of the macro-molecular biosynthetic apparatus with substrates and catalytic components.
Microbiological Reviews. **54**:236-281.

Jensen, M.; Webster, J. and Straus, N. (1993).
Rapid Identification of Bacteria on the Basis of Polymerase Chain Reaction-Amplified Ribosomal DNA Spacer Polymorphisms.
Applied and Environmental Microbiology. **59**:945-952.

Ji, Y.; M.J. Colston and R.A. Cox. (1994).
The ribosomal RNA (*rrn*) operons of fast-growing mycobacteria: primary and secondary structures and their relation to *rrn* operons of pathogenic slow-growers.
Microbiology. **140:**2829-2840.

Jinks-Robertson, S. and M. Nomura. (1987).
Ribosomes and tRNA.
In: *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology. Eds.: F. C. Neidhart; J. L. Ingraham; K. B. Low; B. Magasanik; M. Shaechter and H.E. Umbarger. Published: Washington D.C. 1987.

Johnson, J.L. (1991).
DNA reassociation experiments.
In:Nucleic acid techniques in bacterial systematics (Editors: Stackbrandt, E. and Goodfellow, M.) Published John Wiley and Sons, Chichester.

Johnson, J.L.; Doyle, M.P. and Broderson, P. (1990).
Incidence of *Listeria* spp. in retail meat roasts.
Journal of Food Science. **55**:572-574.

Jones D. and Seeliger H.P.R. (1982).
International Committee on Systematic Bacteriology-Subcommittee on the taxonomy of *Listeria, Brochothrix* and *Erysipelothrix*. Minutes of the meeting, September 7-8, 1986, Manchester.
International Journal of Systematic Bacteriology. **37**:176

Jones, C.E.; Shama, G.; Andrew, P.W.; Roberts, I.S. and Jones, D. (1995).
Comparitive study of the growth of *Listeria monocytogenes* in defined media and demonstration of growth in continuous culture.
Journal of Applied Bacteriology. **78**:66-70.

Jukes, T. and Cantor, C. (1969).
Evolution of protein molecules.
In: Mammalian protein metabolism. (Ed. H. Munro). Published: Academic Press, Inc. New York.

Juntilla J.R.; Niemala, S.I.; Hirn, J. (1988).
Minimum growth temperature of *Listeria moncytogenes* and non-haemolytic *Listeria*.
Journal of Applied Bacteriology. **65**:321-327.

Kane, M.D.; Poulsen, L.K. and Stahl, D.A. (1993).
Monitoring the Enrichment and Isolation of Sulfate-Reducing Bacteria by Using Oligonucleotide Hybridisation Probes Designed from Environmentally Derived 16S rRNA Sequences.
Applied and Environmental Microbiology. **59**:682-686.

Kaplan, R. and Apirion, D. (1974).
The Involvelement of Ribonuclease I, Ribonuclease II and Polynucleotide Phosphorylase on the Degredation of Stable Ribonucleic Acid during Carbon Starvation in *Escherichia coli*.
Journal of Biological Chemistry. **249**:149-151.

Kaplan, R. and Apirion, D. (1975).
The Fate of Ribosomes in *Eschericia coli* Cells Starved for a Carbon Source.
Journal of Biological Chemistry. **250**:1854-1863.

Kaprelyants, A.S. and Kell, D.B. (1992).
Rapid assessment of bacterial viability and vitality by rhodamine 123 and flow cytometry.
Journal of Applied Bacteriology. **72**:410-422.

Kathariuo, S.; Mizumoto, C.; Allen, R.D.; Fok, A.K. and Benedict, A.A. (1994).
Monoclonal Antibodies with a High Degree of Specificity for *Listeria monocytogenes* Serotype 4b.
Applied and Environmental Microbiology. **60**:3548-3552.

Kemp, J.D. (1993).
The role of iron and iron-proteins in lymphocyte physiology and pathology.
Journal of Clinical Immunology. **13**:81-92.

Kemp, P.F., Lee, S.; and LaRoche, J. (1993).
Estimating the Growth Rate of Slowly Growing Marine Bacterium from rRNA Content.
Applied and Environmental Microbiology **59:**2594.

Kerkhof, L. and Ward, B.B. (1993).
Comparison of Nucleic Acid Hybridisation and Fluorometry for Measurement of the Relationship between RNA/DNA Ratio and Growth Rate in a Marine Bacterium.
Applied and Environmental Microbiology. **59**:1303-1309.

Kerr, K.G.; Rotowa, N.A.; Hawkey, P.M. and Lacey, R.W. (1990).
Evaluation of the Mast ID and API 50CH systems for identification of *Listeria spp.*
Applied and Environmental Microbiology. **56**:657-660.

Kihm, D.J.; Leyer, G.J.; An, G.-H. and Johnson, E.A. (1994).
Sensitisation of Heat-Treated *Listeria monocytogenes* to Added Lysozyme in Milk.
Applied and Environmental Microbiology. **60**:3854-3861.

King, W.; Raposa, S.; Warshaw, J.; Johnson, A.; Halbert, D. and Klinger, J.D. (1989).
A new colorimetric nucleic acid hybridisation assay for *Listeria* in foods.
International Journal of Food Microbiology. **8**:225-232.

Kiss, A.; B. Sain, and P. Venetiamer. (1977).
The number of rRNA genes in *Escherichia coli.*
FEBS Letters. **79:**77-79.

Kjellgaard, N.O. and Kurland, C.G. (1963).
The Distribution of Soluble and Ribosomal RNA as a Function of Growth Rate.
Journal of Molecular Biology. **6**:341-348.

Ko, R.; Smith, L.T. and Smith, G. (1994).
Glycine Betaine Confers Enhanced Osmotolerance and Cryotolerance on *Listeria monocytogenes.*
Journal of Bacteriology. **176**:426-431.

Kramer, J.G. and Singleton, F.L. (1992).
Variations in rRNA Content of Marine *Vibrio spp.* during Starvation-Survival and Recovery.
Applied and Environmental Microbiology. **58**:201-207.

Lamont, R.J.; Postlethwaite, R. and MacGowan, A.P. (1988).
Listeria monocytogenes and its role in human infection.
Journal of Infection. **17**:7-28.

Lane, D.J. (1991).
16S/23S rRNA Sequencing.
In: Nucleic Acid Techniques in Bacterial Systematics.
Eds: Stackebrandt, E. and Goodfellow, M.
Published John Wiley and Sons, Chichester, England, 1991.

Lantz, P.-G.; Tjerneld, F.; Borch, E.; Hahn-Huägerdahl, B. and Rådström, P. (1994).
Enhanced sensitivity in PCR detection of *Listeria monocytogenes* in soft cheese through use of an aqueous two-phase system as a sample preparation method.
Applied and Environmental Microbiology. **60**:3416-3418.

Lawrence, L. and Gilmour, A. (1994).
Incidence of *Listeria spp.* and *Listeria monocytogenes* in a Poultry Processing Environment and in Poultry Products and Their Rapid Confirmation by Multiplex PCR.
Applied and Environmental Microbiology. **60**:4600-4604.

LeBaron, P. and Joux, F. (1994).
Flow Cytometric Analysis of the Cellular DNA Content of *Salmonella typhimurium* and *Alteromonas haloplanktis* during Starvation and Recovery in Seawater.
Applied and Environmental Microbiology. **60**:4345-4350.

LeFauci, G; Widom, R.; Eisner, L.; Jarvis, E.D. and Rudner, R. (1986).
Mapping of rRNA genes with integrable plasmid in *Bacillus subtilis.*
Journal of Bacteriology. **165:**5523-5529.

LePecq, J. and Paoletti, C. (1966).
A New Fluorometric Method for RNA and DNA Determination.
Analytical Biochemstry. **17:**100-107.

Leser, T.D.; Boye, M. and Hendriksen, N.B. (1995).
Survival and Activity of *Pseudomonas* spp. Strain B13(FR1) in a Marine Microcosm Determined by Quantitative PCR and an rRNA-Targeting Probe and Its Effect on the Indigenous Bacterioplankton.
Applied and Environmental Microbiology. **61**:1201-1207.

Liesack, W.; Weyland, H. and Stackebrandt,E. (1991).
Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed-culture of strict barophilic bacteria.
Microbial Ecology. **21:** 191-198.

Linton, R.H. ;Carter, W.H.; Pierson, M.D. and Hackney, C.R. (1995).
Use of a Modified Gompertz Equation to Model Nonlinear Survival Curves for *Listeria monocytogenes* Scott A.
Journal of Food Protection. **58**:946-954.

Loessner, M.J.; Schneider, A. and Scherer, S. (1995).
A New Procedure for Efficient Recovery of DNA, RNA and Proteins from *Listeria* cells by Rapid Lysis with a Recombinant Bacteriophage Endolysin.
Applied and Environmetal Microbiology. **61**:1150-1152.

Lynch, J.M. and Hobbie, J.E. (1988).
Micro-organsims in Action: Concepts and Applications in Microbial Ecology.
Blackwell Scientific Publications,Oxford. 1988.

Macfarlane D.E. and C.E. Dahle. (1993).
Isolating RNA from blood -the dawn of RNA based diagnosis?
Nature. **362**:186-188.

Maciukenas, M. (1991).
Treetool Computer Program.
University of Illinois Board of Trustees, Urbana, USA.

McClure, P.J.; Roberts, T.A. and Otto-Oguru, P. (1988).
Comparison of the effects of sodium chloride, pH and temperature on the growth of *Listeria monocytogenes* on gradient plates and in liquid medium.
Letters in Applied Microbiology. **9**:95-99.

McKay, A.M. (1993).
The effect of temperature on culturability and detection of *Listeria innocua* in water.
Letters in Applied Microbiology. **17**:185-187.

McKellar, R.C. (1994).
Use of the CAMP Test for Identification of *Listeria monocytogenes*.
Applied and Environmental Microbiology. **60**:4219-4225.

McLauchlin, J.A.; Audidrier, A. amd Taylor, A.G. (1986).
The Evaluation of a Phage Typing System for *Listeria monocytogenes* for use in epidemiological studies.
Journal of Medical Microbiology. **22**:357-365.

McSweeney, C.S.; Mackie, R.I.; Odenyo, A.A. and Stahl, D.A. (1993).
Development of an Oligonucleotide Probe Targeting 16S rRNA and Its Application for Detection and Quantitation of the Ruminal Bacterium *Synergistes jonesii* in a Mixed-Population Chemostat.
Applied and Environmental Microbiology. **59**: 1607-1612.

Mafu, A.A.; Roy, D.; Goulet, J. and Magny, P. (1990).
Attachment of *Listeria monocytogenes* to Stainless Steel, Glass, Polypropylene, and Rubber Surfaces After Short Contact Times.
Journal of Food Protection. **53**:742-746.

Maidak, B.L.; Larsen, N.; McCaughey, M.J.; Overbeek, R.; Olsen, G.J.; Fogel, K.; Blandy, J. and Woese, C.R. (1994).
The Ribosomal Database Project.
Nucleic Acids Research. **22**:3485-3487.

Makino, S.-I.; Okada, Y. and Maruyama, T. (1995).
A New Method for Direct Detection of *Listeria monocytogenes* from Foods by PCR.
Applied and Environmental Microbiology. **61**:3745-3747.

Markovits, J.; Roques, B.P. and LePecq, J.-B. (1979).
Ethidium Dimer: A New Reagent for the Fluorometric Determination of Nucleic Acids.
Analytical Biochemistry. **94**:259-264.

Marquet-van der Mee, N. and Audidrier, A. (1995).
Proposals for Optimisation of the International Phage Typing System for *Listeria monocytogenes*: Combined Analysis of Phage Lytic Spectrum and Variability of Typing Results.
Applied and Environmental Microbiology. **61**:303-309.

Matseyuma, T. (1984).
Staining of living bacteria with rhodamine 123.
FEMS Microbiology Letters. **21**:153-157.

Mattingly, J.A.; Butman, B.T.; Plank, M.C. Durham, R.J. and Robinson, B.J. (1988).
Rapid monoclonal antibody-based enzyme-linked immunosorbent assay for detection of *Listeria* in food products.
Journal of Official Analytical Chemistry. **71**:679-681.

Mendonca, A.F. and Knabel, S.J. (1994).
A novel Strictly Anaerobic Recovery and Enrichment System Incorporating Lithium for Detection of Heat-Injured *Listeria monocytogenes* in Pasteurized Milk Containing Background Microflora.
Applied and Environmental Microbiology. **60**:4001-4008.

Mengaud, J.C.; Braunbreton, C. and Cossart, P. (1991).
Identification of phosphatidylinositol-specific phospholipase-C activity in *Listeria monocytogenes*- a novel type of virulence factor.
Molecular Microbiology. **5**:367-372.

Mengaud, J.; Gormley, E.; Vincent, M.F.; Chevevert, J.; Baquero, F.; Perez-Diaz, J.C. and Cossart, P. (1990).
Listeriolysin O gene: role in virulence and use as a DNA probe.
In: Foodborne Listeriosis: Eds. A.J. Miller; J.L. Smith and G.A. Somkuti. 1990. Society for Industrial Microbiology.

Mengaud, J.; Vicente, M.-F.; Chenevert, J.; Perreira, J.M.; Geoffroy, C.; Gicquel-Sanzey, B.; Bequero, F.; Perez-Diaz, J.-C. and Cossart, P. (1988).
Expression in *Eschericia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes*.
Infection and Immunity. **56**:766-772.

Michard, J. and Jardy, N. (1989)
Denombrement et localisation de *Listeria monocytogenes* dans des fromages a pate, molle et a croute lavee fabriques avec du lait cru en prevenance d'une enterprise fromagere.
Microbiolgie Alimenteries et Nutrition. **7**:131-137.

Michel E. and Cosssart, P. (1992).
Physical Map of the *Listeria monocytogenes* Chromosome.
Journal of Bacteriology. **174**:7098-7103.

Miles, C.A. and Mackey, B.M. (1994).
A mathematical analysis of microbial inactivation at linearly rising temperatures: calculation of the temperature rise needed to kill *Listeria monocytogenes* in different foods and methods for dynamic measurements of D and z values.
Journal of Applied Bacteriology. **77**:14-20.

Monod, J. (1949).
The Growth of Bacterial Cultures
Annual Review of Microbiology. **3**:371.

Moller, S.; Kristiansen, C.S.; Poulsen, L.K.; Carstensen, J.M. and Molin, S. (1995).
Bacterial Growth on Surfaces: Automated Image Analysis for Quantification of Growth Rate-Related Parameters.
Applied and Environmetal Microbiology. **61**:741-748.

Moyer, C. L.; Mordy, C.W.; Carlson, D.J. and Morita, R.Y. (1990).
Ethidium homodimer used for the sensitive measurement of DNA and RNA of a psychrophilic marine bacterium grown at different growth rate during starvation-survival.
Journal of Micorbiological Methods. **12**:75-81.

Muirhead, K.A.; Horan, P.K. and Poste, G. (1985).
Flow Cytometry: Present and Future.
Bio/technology. **3**:337-356.

Mukhopadhyay, T. and Roth, J.A. (1993).
Silicone Lubricant Enhances Recovery of Nucleic Acids after Phenol-Chloroform Extraction.
Nucleic Acids Research. **21:**781-782.

Munk, M.E. and Kaufmann, S.H.E. (1988).
Listeria monocytogenes reactive T lymphocytes in healthy individuals.
Microbiology and Pathology. **5**:49-54.

Musgrove, E.A. and Hedley, D.W. (1990).
Measurement of intracellular pH.
Methods in Cellular Biology. **33**:59-69.

Nakayama, H.; Yokoi, H. and Fujita, J. (1992).
Quantification of mRNA by non-radioactive RT-PCR and CCD imaging system.
Nucleic Acids Research. **20**:4939.

Neiderhauser, C.; Candrian, U.; Höfelein, C.; Jermini, M.; Bühler, H.-P.; and Lüthy, J. (1992).
Use of Polymerase Chain Reaction for Detection of *Listeria monocytogenes* in Food.
Applied and Environmental Microbiology. **58:**1564-1568.

Neiderhauser, C.; Hofelein, C.; Allmann, M.; Burkhalter, P.; Luther, J. and Candrian, U. (1994).
Random amplification of polymorphic bacterial DNA: evaluation of 11 oligonucleotides and application to food contaminated with *Listeria monocytogenes*.
Journal of Applied Bacteriology. 77:574-582.

Neidhardt, F.C.; Ingraham, J.L. and Schaecter, M. (1990).
Physiology of the Bacterial Cell: A Molecular Approach.
Published: Sinauer Associates, Sunderland, Massachusetts, USA.

Nguyen-the, C. and Lund, B.M. (1991).
The lethal effect of carrots on *Listeria* species
Journal of Applied Bacteriology. **70**:479-488.

Nguyen-the, C. and Lund, B.M. (1992)
An investigation of the antibacterial effect of carrot on *Listeria monocytogenes*.
Journal of Applied Bacteriology. **73**:23-30.

Nocera, D.; Bannerman, E.; Rocourt, J.; Jaton-Ogay, K. and Bille, J. (1990).
Characterisation by DNA restriction endonuclease analysis of *Listeria monocytogenes* strains related to the Swiss epidemic of listeriosis.
Journal of Clinical Microbiology. **28**:2259-2263.

Noll, M. and Noll, H. (1976).
Structural Dynamics of Bacterial Ribosomes: V; Magnesium dependant Dissociation of Tight Couples into Subunits: Measurements of Dissociation Constants.
Journal of Molecular Biology. **105**:111-130.

Nørrung, B. and Skovgaard, N. (1993).
Application of multilocus enzyme electrophoresis in studies of the epidemiology of *Listeria monocytogenes* in Denmark.
Applied and Environmental Microbiology. **59**:2817-2822.

Page, S. and Burns, R.G. (1991).
Flow Cytometry as a Means of Enumerating Bacteria Introduced into Soil.
Soil Biology and Biochemistry. **23**:1025-1028.

Parmar, N.; Easter, M.C.; Forsythe, S.J. (1992).
The detection of *Salmonella enteriditis* and *Salmonella typhimurium* using immunomagnetic separation and conductance microbiology.
Letters in Apllied Microbiology. **15**:175-178.

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

Parry, S.H.; Briggs, T.; Blades, J.A.; Gani, M. and Piron, J. (1990).
The Rapid Clearview™ *Listeria* immunoassay for Detection of *Listeria* species.
Unilver Research Section, Colworth Laboratory, Sharnbrook, Bedfordshire.

Patel, J.R. and Beuchat, L.R. (1995).
Evaluation of enrichment broths for their ability to recover heat-injured *Listeria monocytogenes*.
Journal of Bacteriology. **78**:366-372.

Payne, M.J.; Campbell, S. and Kroll, R.G. (1992).
The use of immobilized lectins in the separation of *Staphylococcus aureus*, *Escherichia coli, Listeria and Salmonella spp.* from pure cultures and foods.
Journal of Applied Bacteriology. **73**:41-52.

Pedras-Vasconcelos, J.A. and Denner, H. (1994).
Expression of Superoxide Dismutase in *Listeria monocytogenes*.
Applied and Environmental Microbiology. **60**:2360-2366.

Pine, L.; Malcolm, G.B.; Brooks, J.B. and Danshevar, M.I. (1989).
Physiological studies on the growth and utilization of sugars by *Listeria* species.
Canadian Journal of Microbiology. **35**:245-254.

Pini, P.N. and Gilbert, R.J. (1988).
A comparison of two procedures for the isolation of *Listeria monocytogenes* from raw chickens and soft cheeses.
International Journal of Food Microbiology. **7**:331-337.

Pirt, L.J. (1965).
The maintenance energy of bacteria in growing cultures.
Proceedings of the Royal Society, London, Series B. **163**:224.

Porter, J.; Diaper, J.; Edwards, C. and Pickup, R. (1995).
Direct Measurements of Natural Planktonic Bacterial Community Viability by Flow Cytometry.
Applied and Environmental Microbiology. **61**:2783-2786

Portnoy, D.; Chakraborty, A.T.; Goebel, W. and Cossart, P. (1992).
Molecular determinants of *Listeria monocytogenes* pathogenesis.
Infection and Immunity. **60**:1263-1267.

Poulsen, L.K.; Ballard, G.; Stahl, D.A. (1993).
Use of rRNA fluoresence *in situ* hybridisation for measuring the activity of single cells in young and established biofilms.
Applied and Environmental Microbiology. **59**:1354-1360.

Powell, H.A.; Gooding, C.M.; Garrett, S.D.; Lund, B.M. and McKee, R.A. (1994).
Proteinase inhibition of the detection of *Listeria monocytogenes* in milk using the polymerase chain reaction.
Letters in Applied Microbiology. **18**:59-61.

Premaratne, R.J.; Lin, W.-J. and Johnson, E. (1991).
Development of an Improved Chemically Defined minimal medium for *Listeria monocytogenes.*
Applied and Environmental Microbiology. **57**:3046-3048.

Priest, F. and Austin, B. (1993).
Modern Bacterial Taxonomy.
Published: Chapman and Hall, London, 1993.

Ralovich, B.S.; Shahamat, M. and Woodbine, M. (1977).
Further data on the characters of *Listeria* strains.
Medical Microbiology and Immunology. **163**:125-139.

Rasmussen, O.E.; Skouboe, P.; Dons, L.; Rossen, R. and Olsen, J.E. (1995).
Listeria monocytogenes exists in at least three evolutionary lines: evidence from flagellin, invasive associated protein and listeriolysin O genes.
Microbiology. **141**:2053-2061.

Rocourt J. (1988).
Taxonomy of the genus *Listeria.*
Infection **16**(Supplement 2) S89-S91.

Rodriguez, G.G.; Phipps, D.; Ishiguro, K. and Ridgway, H.F. (1992).
Use of a fluorescent redox probe for direct visualisation of actively respiring bacteria.
Applied and Environmental Microbiology. **58**:1801-1808.

Roller, C.; Wagner, M.; Amann, R.; Ludwig, W. and Schleifer, K.-H. (1994).
In-situ probing of Gram-positive bacteria with high G + C content using 23S rRNA targeted oligonucleotides.
Microbiology. **140**:2849-2858.

Rosset, R.; Julien, J. and Monier, R. (1966).
Ribonucleic Acid Composition of Bacteria as a Function of Growth Rate.
Journal of Molecular Biology. **18**:308-320.

Ruhland, G.J.; Hellwig, M.; Wanner, G. and Fiedler, F. (1993).
Cell-surface location of *Listeria*-specific protein p60-detection of *Listeria* cells by indirect immunofluorescence.
Journal of General Microbiology. **139**:609-616.

Ryser, E.T. and Marth, E.H. (1987).
Behaviour of *Listeria monocytogenes* during the manufacture and ripening of cheddar cheese.
Journal of Food Protection. **50**:7-13.

Sambrook, J.E.; E.F. Fritsch and Maniatis, T. (1989).
Molecular cloning: a laboratory manual, 2nd. edition. Pub. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Sardelli, A.D. (1993).
Plateau Effect- Understanding PCR Limitations.
Perkin Elmer Amplifications. Issue **9**:2-5.

Sasahara, K.C. and Zottola, E.A. (1993).
Biofilm Formation by *Listeria monocytogenes* Utilizes a Primary Colonising Microorganism in Flowing Systems.
Journal of Food Protection. **56**:1022-1028.

Schaak, M.M. and Marth, E.H. (1988).
Survival of *Listeria monocytogenes* in refrigerated cultured milks and yoghurt.
Journal of Food Protection. **51**:848-852.

Seeliger H.P.R. (1984).
Modern taxonomy of the *Listeria* group-relationship to its pathogenicity.
Clinical Investigations in Medicine. **7**:217-221.

Seeliger H.P.R. and Hohne, K. (1979).
Serotyping of *Listeria monocytogenes* and related species.
Methods in Microbiology. **13**:31-49.

Seeliger, H.P.R. and Jones, D. (1986).
Genus *Listeria.* p1235-1245. *In*: P.H.A. Sneath, N.S. Mair; M.E. Sharpe and J.G. Holt (editors), Bergeys Manual of Systematic Bacteriology, Vol.2. Published: Williams and Wilkins Co., Baltimore, USA.

Shapiro, H.M. (1990).
Flow Cytometry in Laboratory Microbiology: New Directions.
ASM News. **56**:584-588.

Shehata, T.E. and Marr, A.G. (1970).
Synchronous growth of enteric bacteria.
Journal of Bateriology. **103**:789.

Siddiqui, R. and Khan, M.A. (1989).
Amino acid requirements of six strains of *Listeria monocytogenes.*
Zentrallblatt fur Bakteriologie. **271**:146-152.

Siebert, P.D. and Larrick, J.W. (1992).
Competitive PCR.
Nature. **359**:557-558.

Siegal, J.L., Hurst, S.F.; Liberman, E.S.; Coleman, S.E. and Bleiweis, A.S. (1981).
Mutanolysin Induced Sheroplasts of *Streptococcus mutans* are True Protoplasts.
Infection and Immunity. **31**:808.

Skerjve, E.; Rørvik, L.M. and Olsvik, Ø.(1990).
Detection of *Listeria monocytogenes* in foods by immunomagnetic separation.
Applied and Environmental Microbiology. **56**:3478-3841.

Slade, P.J. and Collins-Thompson, D.L. (1991).
Differentiation of the genus *Listeria* from other Gram-positive species based on low molecular weight (LMW) RNA profiles.
Journal of Applied Bacteriology. **70**:355-360.

Smith, J.L. (1990).
Stress-induced injury in *Listeria monocytogenes*, p.203-209. *In* Foodborne listeriosis. Society for Industrial Microbiology. Elseiver Science Publishing Inc., New York.

Smith, J.L. and Archer, D.L. (1988).
Heat-Induced Injury in *Listeria monocytogenes*.
Journal of Industrial Microbiology. **3**:105-110.

Smith, J.L. and Buchanan, R.L. (1990).
Identification of supplements that enhance the recovery of *Listeria monocytogenes* on modified Vogel-Johnson agar.
Journal of Food Safety. **10**:155-163.

Smith, J.L. and Hunter, S.E. (1988).
Heat-injury in *Listeria monocytogenes*-prevention by solutes.
Journal of Food Safety. **8**:165-170.

Smith, J. L.; Marmer, B.S. and Benedict, R.C. (1991).
Influence of Growth Temperature on Injury and Death of *Listeria monocytogenes* Scott A During a Mild Heat Treatment.
Journal of Food Protection. **54**:166-169.

Srivastava, A.K. and Schlessinger, D. (1990).
Mechanism and Regulation of Bacterial Ribosomal RNA Processing.
Annual Reviews in Microbiology. **44**:105-129.

ter-Steeg, P.F. and Pieterman, F.H. (1991).
Effects of Aeration conditions, Temperature and pH on Growth rate and Maintenance-Energy Demands of *Listeria innocua* (the physiological substitute of *L. monocytogenes*) in a (Semi-) Chemically Defined Medium.
Confidential Laboratory Progress Report, Unilever Research, Vlaardingen, Netherlands.

Stephens, P.J. and Jones, M.V. (1993).
Reduced ribosomal thermal denaturation in *Listeria monocytogenes* following osmotic and heat shocks.
FEMS Microbiology Letters. **106**:177-182.

Stephens, P.J.; Cole, M.B. and Jones, M.V. (1994).
Effect of heating rate on the thermal inactivation of *Listeria monocytogenes.*
Journal of Applied Bacteriology. **77**:702-708.

Strachan, N.J.C. and Gray, D.I. (1995).
A rapid general method for the identification of PCR products using a fibre-optic biosensor and its application to the detection of *Listeria.*
Letters in Applied Microbiology. **21**:5-9.

Sulzer, G. and Busse, M. (1991)
Growth inhibition of *Listeria spp.* on Camembert cheese by bacteria producing inhibitory substances.
International Journal of Food Microbiology. **14**:289-296.

Suzuki, H.J. and Giavanonni, S.J. (1996).
Bias Caused by Template Annealing in the Amplification of Mixtures of 16S rRNA Genes by PCR.
Applied and Environmental Microbiology. **62**:625-630.

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

Verheul, A.; Hagting, A.; Amezaga, M.-R.; Booth, I.R.; Rombouts and Abee, T. (1995).
A Di- and Tripeptide Transport System Can Supply *Listeria monocytogenes* Scott A with Amino Acids Essential for Growth.
Applied and Environmental Microbiology. **61**:225-233.

Verheul, A.; Rombouts, F.M.; Beumer, R.R.; Abee, T. (1995)
An ATP Dependant L-Carnitine Transporter in *Listeria monocytogenes* Scott A is Involved in Osmoprotection.
Journal of Bacteriology. **177**:3205-3212.

Wagner, R. (1994).
The regulation of ribosomal RNA synthesis and bacterial cell growth.
Archives of Microbiology. **161:**100-109.

Walker, S.J. and Stringer, M.F. (1987).
Growth of *Listeria monocytogenes* and *Aeromonas hydrophila* at chill temperatures.
Journal of Applied Bacteriology. **63**:R20.

Wallace, B.; Shaffer, J.; Murphy, R.F.; Bomer, J.; Mirise, T. and Itakua, K. (1979).
Hybridisation of synthetic oligonucleotides to ϕX 174 DNA: the effect of single base pair mismatch.
Nucleic Acids Research. **6**:3543-3557.

Wallner, G.; Amann, R. and Beisker, W. (1993).
Optimizing fluoresent in-situ hybridisation of suspended cells with rRNA-targeted oligonucleotide probes for the flow cytometric identification of microorganisms.
Cytometry. **14**:136-143.

Wang, R.-F.; W.-W. Cao; M.G. Johnson. (1991).
Development of a 16S rRNA-Based Oligomer Probe Specific for *Listeria monocytogenes.*
Applied and Environmental Microbiology. **57:**3666-3670.

Wang, R.-F.; Cao., W.-W. and Johnson , M.G. (1992).
16S rRNA-Based Probes and Polymerase Chain Reaction Method to Detect *Listeria monocytogenes* Cells added to Foods.
Applied and Environmental Microbiology. **58**:2827-2831.

Waterhouse, R.N. and Glover, L.A. (1993).
Differences in the Hybridisation Pattern of *Bacillus subtilis* Genes Coding for rRNA Depend on the Method of DNA Preparation.
Applied and Environmental Microbiology. **59**:919-921.

Welshimer, H.J. (1963).
Vitamin requirements of *Listeria monocytogenes.*
Journal of Bacteriology. **85**:1156-1159.

Werners, K.; Heuvelman, C.J.; Chakroborty, T. and Notermans, S.H.W. (1991).
Use of the polymerase chain reaction for direct detection of *Listeria monocytogenes* in soft cheese.
Journal of Applied Bacteriology. **70:**121-126.

Wiedman, M.; Barany, F. and Batt, C.A. (1993).
Detection of *Listeria monocytogenes* with a Nonisotopic Polymerase Chain Reaction-Coupled Ligase Chain Reaction Assay.
Applied and Environmental Microbiology. **59:**2743-2745.

Wiesner, R.J. (1992).
Direct quantification of picomolar concentrations of mRNAs by mathematical analysis of a reverse transcription/ exponential polymerase chain reaction assay.
Nucleic Acids Research. **20**:5863-5864.

Wilson, H.A. and Chusel, T.M. (1985).
Lymphocyte membrane potential and Ca^{2+}- sensitive potassium channels described by oxonol dye fluorescence.
Journal of Cellular Physiology. **125**:72-81.

Winkowski, K.; Crandall, A. and Montville, T.J. (1993).
Inhibition of *Listeria monocytogenes* by *Lactobacillus bavaricus* MN in Beef Systems at Refrigeration Temperatures.
Applied and Environmental Microbiology. **59**:2552-2557.

Woese, C.R.; Gutell, R.; Gupta, R. and Noller, H.F. (1983).
Detailed analysis of the higher-order structure of 16S-like ribosomal ribonucleic acids.
Microbiology Reviews. **47**;621-669.

Wright, P.A. and Wynford-Thomas, D. (1990).
The Polymerase Chain Reaction: Miracle or Mirage? A Critical Review of its uses and Limitations in Diagnosis and Research.
Journal of Pathology. **162**:99-117.

Yang, B.; Yolken, R. and Viscidi, R. (1993).
Quantitative Polymerase Chain Reaction by Monitoring Enzymatic Activity of DNA Polymerase.
Analytical Biochemistry. **208**:110-116.

Young, B.D. and Anderson, M.L.M. (1985).
Quantitative Analysis of Solution Hybridisation.
In: Nucleic Acid Hybridisation: a Practical Approach.
Eds. B.D. Hames & S.J.Higgins. Published IRL Press, Oxford 1985.

Young, K.M. and Fogeding, P.M. (1993).
Acetic, lactic and citric acids and pH inhibition of *Listeria monocytogenes* ScottA and the effect on intracellular pH.
Journal of Applied Bacteriology. **74**:515-520.

Yu, K. and Pauls, K.P. (1992).
Optimisation of the PCR program for RAPD analysis.
Nucleic Acids Research. **20**:2606.

Zottola, E.A. and Sasahara, K.C. (1994).
Microbial biofilms in the food processing industry- Should they be a concern?
International Journal of Food Microbiology. **23**:125-148.