Physiological and biochemical studies on psychrotolerance in *Listeria monocytogenes.*

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by

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STATEMENT

The work in this thesis was carried out by the author, unless otherwise stated in the text, during the period October 1992 to December 1995, under the supervision of Professor P.W. Andrew, Dr. G. Shama[1], Dr. Dorothy Jones and Dr. I.S. Roberts in the Department of Microbiology and Immunology, University of Leicester and [1]Department of Chemical Engineering, Loughborough University. This thesis is submitted for the degree of Doctor of Philosophy at Leicester University and has not been submitted in full or part for any other degree.

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C.E. Jones.

ABSTRACT

Listeria monocytogenes is known to survive and multiply at refrigeration temperatures. Although this behaviour is of concern to the food industry, detailed knowledge of how the organism can sustain growth at these low temperatures is limited.

Previous studies have indicated that changes in fatty acid content and carbohydrate uptake rates may be factors in the ability of the organism to grow at low temperatures (Puttman *et al.*, 1993; Annous *et al.*, 1995, Wilkins *et al.*, 1972). However, these studies used cells grown in batch culture using a complex medium so that the effects of growth rate could not be separated from those of temperature. In addition, it is known that microorganisms are able to acquire lipids from complex growth media (Ratledge and Wilkinson, 1985).

In order to overcome these problems, the physiological effects of changes in the growth temperature were investigated using cells of *L. monocytogenes* grown in a chemostat at a single growth rate using a suitable defined medium. Six defined media were compared for their ability to sustain sequential growth of *Listeria monocytogenes* in batch culture. The most suitable of these (Trivett and Meyer, 1971) was then used to culture *L. monocytogenes* NCTC 7973 at a range of growth temperatures between 30°C and 10°C in a chemostat. Fatty acid studies, using continuously cultured cells, indicated that decreasing the growth temperature resulted in a reduction in the amount of *anteiso*-17:0 fatty acid with a concomitant increase in the levels of a number of smaller chain fatty acids.

A qualitative polar lipid study revealed that phosphatidylglycerol and diphosphatidylglycerol, an unidentified phospholipid and two glycolipids were present at all growth temperatures examined. A third glycolipid was seen only at 10°C, the lowest growth temperature examined. A study of carbohydrate uptake using continuously cultured cells indicated that growth at 10°C caused a significant increase in the uptake of 2-deoxy-D-glucose at the highest assay temperature (30°C) only. However, it was also found that assay temperature, rather than growth temperature was the major factor in the rate and amount of 2-deoxy-D-glucose taken up by *L. monocytogenes* NCTC 7973.

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CEJ

For Mum and Dad

CONTENTS

1. INTRODUCTION

Listeria are Gram positive, motile, non-spore forming rods that have a wide distribution in the environment. The genus has six recognised species. These are *Listeria monocytogenes, Listeria ivanovii, Listeria welshimeri, Listeria grayi, Listeria seeligeri,* and *Listeria innocua* (Jones & Seeliger, 1990; Rocourt *et al.*, 1992). Of the six species, two are recognised opportunistic pathogens; *L. monocytogenes* in humans and animals, and *L. ivanovii* in a wide range of animals, but especially sheep (See Gray and Killinger, 1966; Jones and Seeliger, 1990). The disease in both cases is referred to as listeriosis, and has a wide range of clinical symptoms from mild influenza like symptoms, to encephalitis, septicaemia, and abortion in both humans and animals (Gray and Killinger, 1966).

The marked increase in recorded cases of human listeriosis in the 1980s rekindled interest in the genus, and in particular *L. monocytogenes.* This was reflected by the confirmation of the role of contaminated foodstuffs in transmitting listeriosis (Schlech *et al.,* 1983). However, surprisingly little is known about the metabolism of the any of the species, nor the ability of *L. monocytogenes* to exhibit significant growth in foodstuffs at temperatures below 0°C (Walker *et al.,* 1990), a factor of increasing importance in view of the ever increasing sale of cool-stored, ready-prepared foodstuffs. All temperature related physiological studies on the organism have been performed in batch culture, and as a consequence many of the parameters relating to bacterial growth have not been tightly controlled. There is, therefore, a requirement for these studies to be performed using continuous culture where all growth conditions except growth temperature can be standardised.

1.1 HISTORY AND CLASSIFICATION

The organism was first isolated and detailed in 1926 as a small Gram positive rod causing a septicaemic syndrome among laboratory rabbits and guinea pigs in an

animal house in Cambridge University (Murray *et al.*, 1926). The organism was named *Bacterium monocytogenes* because of the marked mononucleosis it caused in the blood of rabbits. A year later, Pirie described a bacterium isolated from the livers of wild gerbils, which he named *Listerella hepatolytica* in honour of Lord Lister, a pioneer in antisepsis (Pirie, 1927a). Pirie later noted that this bacterium was identical to the organism isolated by Murray *et al.* and renamed the organism *Listerella monocytogenes* (Pirie, 1927b). The present day name was adopted in 1940 when Pirie noted that the name *Listerella* had been used previously to describe a mycetozoan, and changed the name to *Listeria monocytogenes* (Pirie, 1940). The name was adopted in the sixth edition of Bergey's Manual of Determinative Bacteriology (Breed *et al.*, 1948), and was approved by the Judicial Commission on Bacteriological Nomenclature and Taxonomy in 1954.

Although the first description of the organism was in 1926, it was almost certainly observed prior to this. For example, Hayem (1891) and Henle (1891) both refer to a similar organism isolated from post mortem examinations of patients who died from listeriosis-type infections. Hulphers, (1911) appears to have been the first worker to have encountered the organism, now known as *L. monocytogenes,* when he isolated an organism from a rabbit's necrotic foot and named it *Bacillus hepatis.* An organism isolated from a case of human meningitis in 1918 and deposited at the Pasteur Institute, Paris by Dumont and Cotoni in 1921, and later identified as *L. monocytogenes* (Cotoni, 1942), is now regarded as the oldest existing strain of the species.

Retrospectively, organisms isolated from cases of listerial infection from a wide range of sources and given names such as *Corynebacterium infantisepticum, Listerella ovis, Listerella cunicula* and *Corynebacterium parvulum* (Gill, 1931; Kankschewa, 1923; Potel, 1950) were proposed as members of the *Listeria* genus.

For the first twenty five years following its initial description, cases of listeriosis were reported extremely rarely, and *L. monocytogenes* was regarded as an animal pathogen. The first confirmed isolation from sheep was made by Gill in 1929 (Gray & Killinger, 1966). Despite sporadic reports of listeric infections in humans (Burn, 1936; Kaplan, 1945) and isolation of the organism from a human infection (Nyfeldt, 1929), the infection was thought to be due to a unique variant of animal infection and was termed *L. monocytogenes var. homanis*. The name was later dropped when the ability of the organism to infect humans became widely recognised (Gray & Killiger, 1966).

1.2 TAXONOMY

1.2.0 Intergeneric taxonomy

Initially *L. monocytogenes* was the only species within the genus *Listeria*, and was classified within the family *Corynebacteriaceae* in the sixth and seventh editions of Bergey's Manual of Determinative Bacteriology (Breed *et al.,* 1948, 1954). By the eighth edition (Buchanan and Gibbons, 1974), however, the status of the genus *Listeria* had been changed to that of a 'genus of uncertain affiliation', its removal from the family *Corynebacteriaceae* was due to several important taxonomic studies: cell wall composition (Cummins and Harris,1956; Schleifer and Kandler, 1971), lipid analysis (Kosaric and Carroll, 1971; Shaw, 1974; Tadayon and Carroll, 1971), nucleic acids (Stuart and Welshimer, 1974) and numerical taxonomy (Davis and Newton, 1969; Davis *et al.,* 1969; Stuart and Pease, 1972; Stuart and Welshimer, 1974). Further studies of the genus using numerical taxonomy (Jones, 1975; Wilkinson and Jones, 1977; Fiedler *et al.,* 1984; Feresu and Jones, 1988) and chemical studies (Collins and Jones, 1981; Kamisango *et al.,* 1982) reinforced the separation of the genus from the *Corynebacteriacae.* 16s rRNA studies confirmed the location of *Listeria* as a distinct taxon in the low G+C Gram positive branch of *Bacillus, Lactobacillus, Clostridium* group, most closely related to *Bronchothrix* (Ludwig *et al.,* 1984).

However analysis of 16s rRNA reverse transcriptase sequences of the genus have indicated that *Listeria* should not be in this family, but in a new distinct one, *Listeriaceae*, containing two genera, *Listeria* and *Bronchothrix* (Collins *et al.*, 1991).

1.2.1 Intrageneric taxonomy

The intrageneric composition of the genus *Listeria* has been subject to continual revision. Numerous taxonomic, morphological, and nucleic acids have shown that the species *Listeria denitrificans,* listed in Bergey's Manual of Systematic Bacteriology (Seeliger and Jones, 1986), as a species *incertae sedis* is not a member of the genus *Listeria* (Collins *et al.,* 1983; Espaze *et al.*, 1986; Fiedler and Seger, 1983; Fiedler *et al.,* 1984; Jones, 1975; Jones *et al.,* 1986; Stuart and Pease, 1972; Stuart and Welshimer, 1973, 1974; Wilkinson and Jones, 1977). Ribosomal nucleic acid studies have clarified the position of *L. denitrificans*, which is now classified in a new genus, *Jonesia* as *Jonesia denitrificans* (Rocourt *et al.,* 1987a).

Many non-pathogenic, non-haemolytic, and markedly β-haemolytic organisms allocated to the species *L. monocytogenes* were initially incorrectly classified, (Rocourt *et al.,* 1982a). The comprehensive DNA-DNA hybridisation work performed by Rocourt *et al.,* (1982a) on a large collection of haemolytic and non-haemolytic strains, designated *L. monocytogenes,* indicated the presence of five separate DNA-DNA homology groups. One group contained the type strain *L. monocytogenes.* The other four contain the non-pathogenic and non-haemolytic strains designated *L. innocua* (Seeliger and Schoofs, 1979; Seeliger, 1981). The markedly β-haemolytic species *L. ivanovii* first described by Ivanov in 1962 (Ivanov, 1975; Seeliger *et al.,* 1984) and the other two groups were later named *L. seeligeri* and *L. welshimeri* (Rocourt and Grimont, 1983). The five genomic groups show a high degree of similarity, when compared using DNA-DNA hybridisation (Rocourt *et al.,* 1982a). Subsequently similarities were shown using

phenotypic, biochemical, and total protein characteristics (Jones *et al.,* 1986; Lamont *et al.,* 1986; Rocourt and Catimel, 1985). Multilocus enzyme electrophoresis have recently reconfirmed this classification and resulted in the detection of two sub-species in *L. ivanovii, L. ivanovii* subspecies *ivanovii* and *L. ivanovii* subspecies *londoniensis*, distinguishable biochemically by their ability to ferment ribose and N-acetyl-D-mannoside (Boerlin *et al.*, 1992).

Listeria grayi and *L. murrayi*, also listed in Bergey's Manual of Systematic Bacteriology (Seeliger and Jones, 1986), as a species *incertae sedis*, have had their status within the genus *Listeria* altered. Chemical and numerical taxonomic studies indicated that the two species were similar to each other and to *L. monocytogenes*, but DNA-DNA hybridisation work (Stuart and Welshimer, 1974) appeared to imply the reclassification of the two species into a new genus, *Murraya.* However the authors also reported that the species were not sufficiently dissimilar to warrant two species within the proposed new genus and designated them *Murraya grayi* subspecies *grayi* and *Murraya grayi* subspecies *murrayi.* Studies by Rocourt *et al.,* (1987a,b) using DNA-DNA hybridisation and 16S rRNA cataloguing indicated a close relationship between the two species and also between *L. murrayi* and *L. monocytogenes*. This was in contrast to work by Feresu and Jones (1988) and also the earlier work by Stuart and Welshimer (1974), who maintained that *L. murrayi* was a strain of *L. grayi.* The view of Feresu and Jones, (1988) has been supported by 16S rRNA reverse transcriptase sequence analysis of their respective type strains, which exhibit a 99.6% similarity (Collins *et al.,* 1991). Recent DNA-DNA hybridisation, multilocus enzyme electrophoresis and rRNA restriction fragment length polymorphism studies have indicated that the two species should now be reconsidered as a single species, *L. grayi* on the grounds of priority (Rocourt *et al.,* 1992).

The advances in taxonomic methods have indicated that there are two distinct lines of descent amongst the respective species within the genus *Listeria* (Table 1.1), (Collins *et al.,* 1991; Rocourt *et al.,* 1982).

1.3 CHARACTERISTICS

1.3.0 Physiological characteristics

Listeria monocytogenes, the type species of the genus, displays a morphology which is common to all members of the genus. Colonies of the organism, incubated for 24 hours at 37°C are 0.5-1.5mm in diameter, round, translucent with a watery consistency and low convex with a finely textured surface and entire margins (Seeliger and Jones, 1986). Removal of aged colonies from agar often leaves an impression and the colonies are often sticky, though they emulsify easily (Gray and Killinger, 1966). When grown on clear solid medium such as tryptose soya agar for 24-36 hours and viewed using a dissecting microscope and obliquely transmitted light (Henry, 1933), as described by Gray (1957), the colonies are seen to have a distinctive blue-green sheen, a characteristic useful to aid identification. The cells are round ended, short, motile (especially at sub-optimal growth temperatures) Gram positive rods, 0.4-0.5μm in diameter and 1-2μm in length occurring either singly or in short chains. Occasionally palisade or Y-forms occur, causing a superficial resemblance to Corynebacteria and microscopic examination also indicates the lack of spores and capsules and also acid-fast negative (Gray and Killinger, 1966; Seeliger and Jones, 1986).

The organism grows over a wide temperature range from as low as -0.5°C (Walker *et al.*, 1990) to 45°C, (Seeliger and Jones, 1986), with an optimum growth temperature of between 30 and 37°C, though strain variation may be evident at temperature extremes, and many strains will not multiply at temperatures above 41-42°C. Flagella are expressed only when the organism is

Table 1.1 The six species of the genus *Listeria*, their subspecies and the two lines of descent.

	Species	Subspecies
	L. monocytogenes	
	L. ivanovii	*ivanovii, londoniensis*
Line one	*L. seeligeri*	
	L. innocua	
	L. welshimeri	
Line two	*L. grayi*	

grown in the temperature range 20-25°C and are predominately peritrichous. The flagellae are reversibly damaged at temperatures above 30° C and motility is lost (Seeliger, 1961).

Listeria monocytogenes is reported to grow at pH values between 5.6-9.6 (Seeliger and Jones, 1986), with maximum growth achieved at neutral or slightly alkaline pH. George *et al.,* (1988) and Farber *et al.*, (1989) reported growth at 30°C at pH values as low as 4.3-4.4. Both groups of workers also showed that a decrease in growth temperature increased the minimum growth pH, and Farber *et al.,* (1989) also showed that some acidulants (e.g. acetic acid) were more effective in inhibiting growth.

Extended survival and growth of *L. monocytogenes* in a wide range of salt concentrations has been observed, with the organism able to withstand up to 150 days in pure salt at ambient temperatures (Von Sielaff, 1968). Seeliger and Jones, (1986) reported that *L. monocytogenes* is able to grow in nutrient broth containing up to 10% (w/v) NaCl, though strain variation was seen. Ryser and Marth, (1991) reported the ability of the organism to withstand NaCl concentrations of up to 12% in brain heart infusion. However exposure to high salt concentrations appears to lead to morphological changes, the cells may become elongated or filamentous as cell division is inhibited without cessation of cell growth (Ryser and Marth, 1991). Increased halotolerance appears to occur when the organism is in salted meat, Von Sielaff (1968) detected *L. monocytogenes* in beef immersed in 22% (w/v) NaCl after 100 days. The role of compatible solutes such as glycine betaine, proline and carnitine in conferring a degree of osmotolerance at high NaCl concentrations has been shown for *L. monocytogenes* (Patchett *et al.*, 1992, 1994; Beumer *et al.,* 1994, Giffel, 1995). These studies indicated that growth of the organism in medium containing up to 5% (w/v) NaCl is enhanced if, notably glycine betaine, but also proline, carnitine, and choline are also present in the medium. The organism is unable to synthesise

glycine betaine, and the mechanisms by which osmoprotectants work are thought to be similar to those for cryoprotectants [See 1.9]. Beumer *et al.*, (1994) Amezaga *et al.,* (1995) looked at the respective osmoprotectant qualities of a complex and a defined medium and showed that the complex medium (brain heart infusion broth (BHI)) was more able to support growth of *L. monocytogenes* in the presence of increasing concentrations of NaCl. The presence of potential osmoprotectants within BHI was studied in detail by Amezaga *et al.*, (1995) who showed that the medium contained quantities of glycine betaine and also peptone. When present in a medium, peptone provides a source of amino acids and peptides which can act in a turgor protecting role. Amezaga *et al.*, (1995) showed that, proline- and glycine- containing peptides were able to stimulate growth at high osmolarity.

1.3.1 Biochemical characteristics

Listeria monocytogenes is catalase positive, Voges-Proskauer test positive, asculin hydrolysed, alkaline phosphatase positive, urease negative, and oxidase negative (Rocourt and Catimel, 1985; Seeliger and Jones, 1986). The genus is facultatively anaerobic and produces acid without gas from glucose and other carbohydrates. The ability of the respective species in the genus *Listeria* to produce acid from a range of carbohydrates can aid their identification (Table 1.2). Other distinguishing tests are shown in Table 1.3. The CAMP test (Christie *et al.,* 1944) is performed by streaking cultures of *Staphylococcus aureus* and *Rhodococcus equi* vertically on sheep blood agar. Colonies of *Listeria* are then streaked at right angles to the other cultures. After incubation at 35°C for 48 hours, β-haemolysis produced by *L. monocytogenes* and *L. seeligeri* is enhanced by the β-toxin of *S. aureus*. Haemolysis of *L. ivanovii* is not enhanced by the β-toxin of *S. aureus,* but by an exosubstance of *R. equi*. The other species of *Listeria* do not produce any positive reaction in the CAMP test (Table 1.3).

Table 1.2 Acid production from carbohydrates.

Acid production from	*L. monocytogenes*	*L. innocua*	*L. ivanovii*	*L. seeligeri*	*L. welshimeri*	*L. grayi*
Gluconate	-	-	-	-	-	+
Lactose	d	+	+	+	+	+
D-Lyxose	d	d	-	d	d	-
Mannitol	-	-	-	-	-	+
Melezitose	d	+	d	d	d	-
α-Methyl-D-glucoside	+	+	+	+	+	+
α-Methyl-D-mannoside	+	+	-	-	+	+
L-Rhamnose	+	d	-	-	d	-
Ribose	d	d	+	d	d	+
Saccharose	d	d	d	d	+	-
D-Tagatose	d	d	-	d	d	-
D-Turanose	d	d	d	d	d	-
D-Xylose	-	-	+	+	+	-

+, 90% or more of strains positive; -, 90% or more of strains negative; d, 11-89% strains positive

Adapted from Jones and Seeliger, (1990).

Table 1.3 Abbreviated Biochemical Identification of *Listeria* species.

		CAMP test	
Listeria species	β Haemolysis	*S. aureus*[a]	*R. equi*[b]
L.monocytogenes	+	+	-
L. ivanovii	+	-	+
L. innocua	-	-	-
L. seeligeri	V	+	-
L. welshimeri	-	-	-
L. grayi	-	ND	ND

[a] *S. aureus* NCTC 1803; [b] *R. equi* NCTC 1621; ND Not Determined; V Variable.

Adapted from Ryser and Marth, (1991).

1.3.2 Serology

Listeria monocytogenes can be divided serologically into four serological types on the basis of their somatic (O) and flagella (H) antigens (Paterson, 1940). The current scheme, extended by Donker-Voet, (1957); Seeliger, (1953); Seeliger and Schoofs, (1979), and Seeliger and Hohne, (1979) is known as Seeliger-Donker-Voet Scheme and gives sixteen serovars amongst five antigenically similar species (Table 1.4). *L. grayi* and *L. grayi* subspp *murrayi* are antigenically different from the other species, but closely related to each other. However, with the exception of serovar 5 strains, all of which belong to the species *L. ivanovii*, there is not a definite relationship between serovar and species. *L. seeligeri* cannot be distinguished serologically from *L. monocytogenes* and *L. welshimeri* cannot be distinguished serologically from *L. innocua* serotype 6b (Table 1.4).

Table 1.4 Serotypes of *Listeria* species.

Listeria species	Serotype	O antigens present	H antigens present
L. monocytogenes	1/2a	I II (III)*	AB
	1/2b	I II (III)	ABC
	1/2c	I II (III)	BD
	3a	II (III) IV	AB
	3b	II (III) IV (XII) (XIII)	ABC
	3c	II (III) IV (XII) (XIII)	BD
	4a	(III) (V) VII IX	ABC
	4ab	(III) (V) VII IX X	ABC
	4b[a]	(III) V VI	ABC
	4c	(III) V VI	ABC
	4d	(III) V VI VIII	ABC
	4e	(III) V VI (VIII) (IX)	ABC
	7	(III) XII XIII	ABC
L. ivanovii	5	(III) (V) VI (VIII) X	ABC
L. innocua	6a	(III) V (VI) (VII) (IX) XV	ABC
	6b	(III) (V) (VI) (VII) IX X XI	ABC
L. grayi		(III) XII XIV [XVI]	E
L. grayi subsp. *murrayi*		(III) XII XIV [XVII]	E

* Indicates not always present; [a] also 4b(x) (McLauchlin *et al.*, 1989).

Adapted from Ryser and Marth, (1991).

1.3.3 Phage typing

Bacteriophages specific for *Listeria* spp. were first discovered in 1945 by Schultz, and several groups have studied the possibility of phage typing for the epidemiological studies. (Rocourt *et al.,* 1985; McLauchlin *et al.,* 1986 Audurier *et al.,* 1984; Audurier and Martin 1989). These early studies however were hampered by the lack of phages available, the overall percentage of *L. monocytogenes* strains typeable ranging from 52-78%. In 1990 however a new set of phage were described by Loessner *et al.,* (1990) These allow the typing of 90% of serotype 1/2, the majority of isolates of serotype 4a, ab, c, d, and e, and although the strains with serotype 3 and 7 appear to be resistant phage typing has been used with success in epidemiological studies of outbreaks of listeriosis (Audurier *et al.,* 1984; Audurier and Martin 1989;). There is currently an international centre for the phage typing of *Listeria* at the Pasteur Institute in Paris, France.

1.3.4 Isoenzyme typing

Isoenzyme typing, a method of differentiating bacteria by the variability in the electrophoretic mobility of any of a large number of metabolic enzymes, has indicated subgroups within serotypes of *L. monocytogenes.* 45 electrophoretic types were identified by Piffaretti *et al.,* (1989), when strains of *L. monocytogenes* were analysed for 16 genetic loci encoding metabolic enzymes. These electrophoretic types could be further divided into two clusters; one containing *L. monocytogenes* strains of serotypes 1/2a and 1/2c, and the other serotypes 1/2b 4a and 4b (Piffaretti *et al.,* 1989). Similar results have been recorded by Bibb *et al.,* (1990), though there were 56 electrophoretic types were identified in this case, with one cluster containing all *L. monocytogenes* strains of flagella H, antigen type a, and another containing all *L. monocytogenes* strains of flagella H, antigen type b examined. More recent multilocus enzyme electrophoresis studies by (Boerlin *et al.,* 1992) indicated strains of *L. ivanovii* are divided into two genomic groups, represented by two subspecies [See 1.2.1].

1.4 ISOLATION

The primary isolation of *L. monocytogenes* from contaminated specimens has long been problematic (Gray and Killinger, 1966). Despite the organism being relatively non-fastidious and able to grow well on nutrient media, a number of methods for the isolation of the organism such as cold enrichment, warm enrichment as well as selective techniques are used for the primary isolation of the organism from both environmental and clinical samples. It seems however, important that prior to any isolation techniques being attempted that the contaminated sample, especially if clinical, must be macerated (Gray and Killiger, 1966).

Cold enrichment (Gray, 1948) is a useful, if somewhat time-consuming method for the isolation of *L. monocytogenes,* making use of the psychrotrophic nature of the organism. The sample is plated onto a non-selective medium and incubated at 4°C for a period usually of about a week but occasionally up to six months. Identification of the organism is by the obliquely transmitted light method of Henry, (1933) [See 1.3.0]. The potentially lengthy time scale required for cold enrichment has led to a wide variety of enrichment techniques being developed. Because of the undoubted success of cold enrichment, it is prudent to proceed with enrichment techniques at both 4°C and 30°C (Jones and Seeliger, 1990). An enrichment medium used extensively for the isolation of *Listeria* is that of Donnelly and Baigent (1986). There are a number of selective media employed, the most successful of which make use of a combination of two inhibitors: nalidixic acid, which inhibits Gram negative bacteria; and acridine dye at a concentration that inhibits many Gram positive bacteria, but not *Listeria.* Selective media incorporating these, and other agents such as potassium tellurite, are commonly used e.g. Oxford and PALCAM media (Curtis *et al.,*1989a; Van Netten *et al.,* 1988). More detailed accounts of the enrichment and selective

media employed for *Listeria* spp and *L. monocytogenes* and also non-conventional methods such as monoclonal antibodies, DNA probes and PCR can be found in Makino *et al.,* (1995); Ryser and Marth, (1991); Farber and Peterkin, (1991); Durham *et al.,* (1990); Jones and Seeliger, (1990); Prentice and Neaves (1988); Datta *et al.,* (1987); and Gray and Killiger, (1966).

1.5 PATHOGENESIS

There are two pathogenic species in the genus *Listeria, L. monocytogenes* (infecting humans and animals) and *L. ivanovii* (infecting animals, mainly sheep). Both are opportunistic, facultative intracellular pathogens and, given that *Listeriae* are widespread in the natural environment [See 1.6], the likelihood of systemic infection is almost entirely dependant on the susceptibility of the individual, the size of the infectious dose and the virulence of the organism.

1.50 Route of entry

Because gastrointestinal problems often preclude the onset of listeriosis, ingestion of the organism is the likeliest port of entry into the body for *L. monocytogenes* though the true minimum infectious dose is unknown. McLauchlin (1995), in an attempt to establish the minimum infective dose for human listeriosis, showed that levels of *L. monocytogenes* in contaminated foods that have caused disease in humans were in excess of 10^3/g. However, in correlating the amount of organism present in an infected foodstuff and a minimum infectious dose, McLauchlin, (1995) advised that caution be applied, because the dose would vary with individual susceptibility and consequently would probably never be known.

Gastrointestinal symptoms occur in approximately one third of listeriosis sufferers and may reflect a non-specific response to a systemic infection

disturbing the mucosal integrity allowing the invasion of *L. monocytogenes* (Bojsen-Moller, 1972; Armstrong, 1985; Gellin and Broome, 1989).

The true infectious dose for other (non-food related) forms of listeriosis are also unknown. (Courcol *et al.,* 1982) showed that *L. monocytogenes* is able to infect the pregnant uterus when very high levels of the organism are present (10^8/ml in amniotic fluid). Similar high levels of the organism would also be present during, and immediately after birth, and be the cause of late onset neonatal infection [See 1.5.1], by a single, large infectious dose to the infant. The similar high levels present during animal birth are also likely to be the cause of cutaneous lesions occasionally seen on farmers and veterinarians [See 1.5.1].

1.5.1 Symptoms of human listeriosis

The clinical manifestations vary widely, ranging from mild, influenza like symptoms, to meningitis and meningoencephalitis. Though rare amongst immunocompetant individuals, the mortality rate amongst infected individuals is relatively high, up to 44% in adults and 50% in neonates (Farber and Peterkin, 1991).

Immunocompromised individuals, including organ transplant and cancer patients in addition to people suffering from Acquired Immune Deficiency Syndrome (AIDS), present the most likely group in the adult population to suffer from listeriosis. Other effected individuals include the very old, the very young and pregnant women. Although at present, uncommon amongst AIDS patients, Gellin and Broome, (1989) estimated that such persons are several hundred times more likely to suffer than healthy members of the population.

In immunocompromised individuals meningitis and bacteraemia/septicaemia are the most likely symptoms, though focal infections such as septic arthritis and liver granulomas also occur (Farber and Peterkin,1989).

Pregnant women are most susceptible to listeriosis whilst in the third trimester of pregnancy. Their classic symptoms are of a mild influenza-like illness, which though unlikely to be life threatening to the mother, is likely to cause infection in the foetus leading to septic abortion. If the mother is diagnosed and treated promptly, by appropriate antibiotics the infection of the foetus can be prevented (Gellin and Broome, 1989; Jones, 1990).

Neonatal listeriosis, can be either early onset, within 2-3 days of birth, with infection occurring *in utero*, or late onset, (>5 days after birth) with infection occurring during or after birth. Early onset listeriosis is characterised by clinical symptoms of pneumonia, bacteraemia, and widespread abscesses. In such cases mortality is high (50%). Neonates developing late onset listeriosis, display clinical symptoms of meningitis only (Gellin and Broome, 1989; Jones, 1990).

Listeric meningitis is the most common clinical condition seen in apparently healthy individuals, but less common infections such as meningoencephilitis, and abscesses of the brain and spinal cord also occur (Gellin and Broome, 1989; Farber and Peterkin, 1991). Central nervous system infection often leads to a series of symptoms including personality changes, tremors, seizures and coma (Neiman and Lorber, 1980). In such cases the organism can be isolated from both the blood and cerebrospinal fluid of infected individuals.

Listeric bacteraemia, is indicated mainly by the presence of fever. Patients suffer from a wide range of symptoms such as nausea, vomiting, diarrhoea, muscle cramps and fatigue (Gellin and Broome, 1989).

In addition to the focal infections seen in immunocompromised individuals, there have been numerous reports of cutaneous lesions, ocular and bronchial infection. These generally milder forms of listeriosis do not usually lead to systemic

infection, though rare cases have occurred, such as the death of a farmer from bronchopneumonia and meningitis probably following inhalation of contaminated faecal matter (Odegaard *et al.,* 1952). Also cases of pulmonary listeriosis in cattle have been traced to contaminated feed (Wouler and Baugh, 1983). Cutaneous listeriosis can be acquired by the handling of infected materials. For example, it has been reported that a veterinarian contracted cutaneous listeric lesions after aiding delivery of an aborted foetus from a *Listeria* infected cow (Owen *et al.,* 1960). The ocular route of infection has been well documented also and cases of conjunctivitis have been reported after accidental eye contamination in the laboratory and also after handling infected chickens (Seeliger, 1961; Ralovich, 1984).

There are a number of anti-microbial agents that are active against *L. monocytogenes in vitro,* these include penicillin G, ampicillin, erythromycin, sulphamethoxazole, rifampin, the tetracyclines and aminoglycosides. However optimal antimicrobial therapy for the various manifestations of listeriosis have not been established clinically (See Gellin and Broome, 1989, Ryser and Marth, 1991).

1.5.2 Symptoms of animal listeriosis

Virtually all domestic and wild animals are susceptible to listeriosis [See 1.6.0]. However the symptoms of the disease given below, refer primarily to animals of importance to humans, namely cows, sheep, pigs and goats.

The clinical manifestations of animal listeriosis are similar to human listeriosis. Sporadic listeriosis in pregnant animals results in abortion and stillbirth, usually in sheep and cattle, though outbreaks amongst pigs have also been recorded (Gitter, 1985). Contaminated faeces is thought to be the agent of sporadic disease. Bacteraemia in animals is most common amongst new-born lambs, often

due to postpartum contamination and symptoms include anorexia, pyrexia, and diarrhoea, (Gitter, 1985; Prentice and Neaves, 1988).

The most prevalent form of animal listeriosis, is encephalitis, resulting in the so-called 'circling disease', first noted by Gill (1931) in sheep. This publication is also the first documented isolation of *L. monocytogenes* from an infected animal. In sheep and goats an incubation of up to three weeks proceeds the onset of encephalitis and the symptoms of circling disease. Other symptoms including raised temperature and a refusal to eat or drink also occur as do neurological disorders such as teeth grinding, paralysis of masticatory muscles and salivation brought about by an inability to swallow. Death, following the onset of these clinical symptoms, occurs rapidly, usually within three days (Ryser and Marth, 1991). Listeriosis in lambs and kids occurs in the form of septicaemia, characterised by a loss of appetite, elevated temperature and diarrhoea, and death can occur due to liver damage and pneumonia, though the death rate is lower than that of the encephalitic form Ryser and Marth, (1991).

Listeriosis in cattle was, in retrospect, first noted by Matthews (1928). The disease is the most common cause of bovine encephalitis and appears to effect beef rather than dairy herds. Unlike sheep or goats, cattle survive for a period of up to 14 days after the onset of clinical symptoms, with a few reports of recovery (Ralovich, 1984). After the onset of clinical symptoms, cattle have been seen to develop additional symptoms such as conjunctivitis, and skin lesions. As the disease progresses, muscular paralysis leads to a protruding tongue and saliva becomes much more viscous (Ryser and Marth, 1991). Less commonly mastitis occurs, causing contamination of the milk for up to threes months after clinical symptoms have ceased. This is of concern to man as contaminated milk is a primary source of human listeriosis [See 1.6.5].

Listeriosis in pigs occurs much less commonly than in sheep, goats and cattle, and though incidences of septicaemia, localised internal abscesses, and skin lesions have been noted, overt forms of listeriosis occur rarely (Ryser and Marth, 1991).

1.5.3 Mechanisms of virulence

The survival and replication of *L. monocytogenes* is dependant on an array of factors that permit invasion and also assist in the evasion of immune defences. A number of factors have been considered as virulence factors in *L. monocytogenes*: notably a haemolysin (listeriolysin O), internalin, and also others such as phospholipase, catalase, superoxide dismutase, and other enzymes, components referred to as monocytosis-producing activity, immunosuppresive activity, delayed type hypersensitivity factor, and the protein p60 (Chakraborty and Goebel, 1988 Sheehan *et al.,* 1994; Dramsi *et al.,* 1996). β-Haemolysis activity on blood agar distinguishes *L. monocytogenes* from non-pathogenic species, however there is little correlation between the amount of haemolysis and the severity of the disease (Groves *et al.,* 1977; Skalka *et al.,* 1982; Parrisius *et al.,* 1986). Transposon mutagenesis has indicated that haemolysin (listeriolysin O) is essential to the virulence of *L. monocytogenes* (Gaillard *et al.,* 1987; Kathariou *et al.,* 1987; Cossart *et al.,* 1989. Listeriolysin O is a sulphydryl activated protein, 59 kDa in length and antigenically similar to streptolysin O (Njoki-Obi, 1963, Parrisius *et al.,* 1986; Gaillard *et al.,* 1987). The production of the haemolysin may also act indirectly as a growth factor by lysing erythrocytes and increasing the amount of available iron to the organism.

Host cell infection of *L. monocytogenes* begins with the internalisation of the organism by phagocytosis in the case of macrophages, or induced phagocytosis, in the case of non-phagocytic cells e.g. intestinal epithelial cells. The adherence and entry of the organism into non-phagocytic is mediated by internalin, a surface protein (Gaillard *et al.,* 1991; Dramsi *et al.,* 1993a,b) and also by InIB (Dramsi *et*

al., 1995, 1996) and (indirectly) by p60 (Dramsi *et al.,* 1996) Once adhered, the bacteria are rapidly incorporated and begin to multiply within the host. Upon phagocytosis, membrane surrounding the phagosome undergoes cytolysis, mediated by listeriolysin, (Cossart and Mengaud, 1989; Sheehan *et al.,* 1994) the organism is then able to multiply freely and rapidly within the cytoplasm. Within a few hours the bacteria is able to induce the polymerisation of actin and become coated with short actin filaments and other actin binding proteins (Tilney and Portnoy, 1989; Tilney *et al.,* 1990; Mournier *et al.,* 1990). This actin structure is then rearranged to form a tail-like structure advancing the bacteria to the cell surface. Propelled by the actin tail, protrusions of the cell membrane form around the listeriae and are pushed into neighbouring cells forming a double membrane which is recognised and internalised. Within the cytoplasm of the new cell the cycle is repeated (Dabiri *et* al., 1990; Tilney *et al.,* 1990; Mournier *et al.,* 1990).

1.6 HABITATS OF *Listeria monocytogenes*

1.6.0 Animals and humans

In addition to humans, *L. monocytogenes* has been isolated from a wide range of animals. Jones and Seeliger (1990), noted that at least 42 species of wild and domestic animals, and 17 avian species are able to harbour the organism. Most domestic animals are susceptible to infection, and, unless treated fatalities are likely to occur. Milder infections in pregnant animals usually leads to damaged, dead or aborted foetus (Seeliger, 1961). The geographical areas where the organism are widespread ranging from the Arctic to Africa (Ryser and Marth, 1991). Despite the increasing frequency with which listeriosis is being diagnosed, the actual extent of infection in animals is unknown, Ralovich, (1984) noted that a steady increase in listeric infections has occurred in some countries of Eastern Europe in the period after 1966. Similar such reports have been mirrored in other countries, for example England, Greece and New Zealand (Ryser and Marth, 1991).

1.6.1 Environmental sources

The ubiquity of *L. monocytogenes* in the natural environment is known (See Ryser and Marth, 1991). Welshimer and Donker Voet, (1971) reported that the primary habitat of the organism is in soil and decaying vegetation, particularly during times in the year when such material provides a moist environment.

1.6.2 Faeces

The presence of *L. monocytogenes* in the faeces of healthy and infected animals has been well documented (See Ryser and Marth, 1991). Ralovich, (1984) also noted the presence of *L. monocytogenes* in both symptomatic and asymptomatic humans of European origin and calculated that the organism is present in between 1.8 and 9% (dependent on age, sex and profession) of the faeces in the healthy human population.

1.6.3 Water

To date, there are no reports of listeriosis from consumption of contaminated water, though there are reports of the presence of the organism in water systems. For example, Dijkstra (1966) reported the presence of the organism in lakes and canal systems in Holland, resulting from the presence of sewage effluent.

1.6.4 Animal feed

In addition to soil and rotting vegetation, another important niche where *L. monocytogenes* has been isolated is animal feed, notably silage. Historically there has been a link between silage and ruminant listeriosis This was confirmed when poor grade silage (silage that after fermentation has a pH >4.5 (Grønstøl, 1979, Fenlon, 1986a, 1986b)), was shown to be the contaminated with *L. monocytogenes* and also was the causative agent of death and abortion in cattle, (Gray, 1960). The incidence of *L. monocytogenes* in silage does appear to be

confined to poorly fermented silage however (Grønstøl, 1979, Fenlon, 1986a, 1986b).

1.6.5 Human food

The link between human listeriosis and food was long suspected. As early as 1915, before the first isolation of the organism, a listeriosis-like disease in 5 young children in Australia was linked with eating of contaminated foodstuffs (Atkinson, 1917). In addition, Pirie, (1927) established a laboratory link between the onset of listeriosis in gerbils and mice with the consumption of feed, artificially contaminated with *L. monocytogenes*. Despite a series of suspected outbreaks of listeriosis in Germany and Czechoslovakia in the years between 1949 and 1957 linked to the consumption of raw milk (Seeliger, 1961; Gray and Killinger, 1966), the connection between listeriosis and the consumption of contaminated foodstuffs was not firmly established until the early 1980s. Since then several outbreaks of human listeriosis have been linked to the consumption of contaminated foodstuffs (Table 1.5). Epidemics causing serious illness or death to a large number of individuals were linked to coleslaw (made from cabbage grown on soil fertilised by sheep faeces obtained from a flock known to have a history of listeriosis), (Schlech *et al.*, 1983); milk, contaminated post-Pasteurisation, (Fleming, *et al.*, 1985); a Swiss cheese ripened in contaminated cellars, (Malinverni *et al.*, 1985) and Mexican ripened (Jalisco) cheese, contaminated with raw milk, (Linnan *et al.*, 1988). Other milk products that have been shown to support the growth of *L. monocytogenes* include chocolate milk, cream and skimmed milk (Rosenow and Marth, 1986).

The publicity generated by these outbreaks have led to a large scale surveys for the presence of the organism in a wide range of foods both with, or without, milk present. Surveys of cooked and uncooked meats, have indicated that the organism is able to survive on the meat surface and in sarcoplasmic protein solutions from meat, (Johnson *et al.*, 1986; Khan *et al.*, 1972;). *Listeriae* have

also been shown to survive cold and warm smoking processes for fish (Fuchs and Nicholaides, 1994). In a study of vacuum packed, cold smoked, salmon inoculated with *L. monocytogenes,* the organism was shown to be able to grow significantly, even under refrigerated conditions, within the shelf life of the product, (Hudson and Mott, 1993).

Poultry has also been implicated as an important vehicle for foodborne listeriosis and chicken carcasses have indicated contamination levels of 33-60% (Kwantes and Isaac, 1972; Pini and Gilbert, 1988).

Despite the presence of *Listeriae* in infected poultry there is little evidence to suggest that the organism is present in eggs. No documented evidence of any outbreaks of human listeriosis have been linked to either eggs, or egg-related products (Dedié, 1955). A recent study (Desmarchelier *et al.,* 1995), analysing possible listerial contamination in the Australian egg industry, showed the presence of quantities of *L. innocua* in 13.4% of environmental samples, and between 100 and 69% of pulped egg, depending upon source. The high values obtained here were due to poorly cleaned machinery and general unsanitary conditions. A raw liquid egg yielded far less contamination (2%), and despite the postulation by the authors that the presence of *L. innocua* provides a marker for the presence of *L. monocytogenes*, the wash of only 1 egg out of 120 tested positive for *L. monocytogenes.*

There is no evidence for the trans-ovarian contamination of listeriosis, but the ability of the organism to grow well in products that contain quantities of eggs implies that there may have, or may well be, cases of listeriosis related to the consumption of products containing contaminated eggs.

Table 1.5 Food borne outbreaks of human listeriosis in North America and Worldwide.

Location (Yr)	No. cases (deaths)	Associated food
Boston (1979)	20 (5)	Salad vegetables[b]
New Zealand (1980)	29 (9)	Shellfish, raw fish[b]
Maritime Provinces (1981)	41 (17)	Coleslaw
Massachusetts (1983)	49 (14)	Pasteurised milk[b]
California (1985)	142 (48)	Jalisco Cheese
Switzerland (1983-87)	122 (31)	Raw milk, cheese
Philadelphia (1986-87)	36 (16)	Ice cream, salami[b]
Connecticut (1989)	9(1)	Shrimp[b]
United Kingdom (1987-89)	>300 (?)	Paté[b]
France (1992)	279 (?)	Pork tongue in jelly
France (1993)	39 (?)	Pork paté

[a] Two of these deaths were due to an underlying disease and not listeriosis.

[b] Foods only epidemiologically linked. ? Exact number of related deaths unknown.

Adapted from Farber and Peterkin, (1991); McLauchlin *et al.,* (1991); Goulet *et al.,* (1995).

In addition to meat and dairy products there has been numerous publications indicating the presence of *Listeriae* in a wide variety of fresh and processed vegetables and associated products such as coleslaw. Given the incidence of animal listeriosis linked to contaminated feed it is reasonable to suspect that raw vegetables are an important vehicle for human listeriosis, as was suggested as early as 1967 by Blendon and Szatalowicz (1967). Ho *et al.,* (1986) indicated that an outbreak of human listeriosis amongst patients in several hospitals in Boston, USA in 1979 was caused by the victims consuming several different foods, of which only salad products, namely contaminated celery, lettuce, and/or tomatoes were eaten by them all. Schlech *et al.,* (1983), reported that coleslaw, containing cabbage contaminated with sheep faeces was the cause of an outbreak in the Canadian Maritime Provinces in 1981, and although in the Boston outbreak, the salad products were the only link between the victims, the vegetables in question were never tested for the presence of *L. monocytogenes.* It is reasonable, however, to postulate that, as with the outbreak in Canada in 1981, any outbreaks with raw vegetable or vegetables as the vehicle for human listeriosis, are likely to have been contaminated by animal faeces containing *L. monocytogenes.* Despite the ability of *L. monocytogenes* to exist on raw vegetables, it has been reported that raw carrots have an inhibitory effect on organism (Beuchatt and Brakett, 1990), the anti-listerial effect is destroyed by heat, however, and further studies to characterise the toxic component remain to be performed.

A report that *L. monocytogenes* was able to survive Pastuerisation was published in 1958 (Bearns and Girard, 1958), however until the mid 1980s human listeriosis had only been linked to raw milk. In 1985, following an outbreak of foodborne listeriosis in Massachusetts, that was linked to the consumption of contaminated Pasteurised milk, the ability of *L. monocytogenes* to survive the Pasteurisation process was again postulated (Fleming *et al.,* 1985). Despite fairly strong epidemiological evidence, however, the microbiological evidence was far less convincing. *Listeria monocytogenes* was never isolated from any of the suspect

Pasteurised milk, nor was the organism isolated post-Pasteurisation, from the incriminated dairy (Fleming *et al.,* 1985). As a result the source of contamination was never conclusively shown. The model used in the work of Bearns and Girard (1958) indicating the ability of *L. monocytogenes* to withstand Pasteurisation has been questioned following the Massachusetts outbreak (Twedt, 1986; Donnelly *et al.,* 1987). Current understanding of Listerial resistance to Pasteurisation is: whilst under laboratory conditions, with high numbers of the organism present in test milk, the organism can survive minimum Pasteurisation requirements, under more true dairy conditions, where contaminated milk would be diluted when pooled with milk from other sources prior to Pasteurisation, both the scientific community and the World Health Organisation contend that the organism is unable to withstand even minimum Pasteurisation requirements. (Ryser and Marth, 1991). The complete failure to find *L. monocytogenes* post-Pasteurisation in dairies, to date, appears to vindicate this position.

There are innumerable food types that are implicated as supporting the growth and survival of *L. monocytogenes* and also several that have led directly to human listeriosis. However in summation, the foods that are of particular danger to susceptible individuals are poultry and associated products; meat and associated products, such as paté, and sausages; seafoods; milk and associated products such as cheeses, and milk drinks, (See Farber and Peterkin, 1991; Ryser and Marth, 1991).

A study of the overall amounts of *L. monocytogenes* isolated from a number of different 'at risk' food types, in the late 1980s to mid 1990s (Table 1.6) in England and Wales, indicate a general fall in the incidence of *L. monocytogenes* in such foods during the latter part of the study, possibly due to improved care and awareness during the manufacturing process (Gilbert, 1995). This fall in incidence of *L. monocytogenes* in 'at risk' foods indicated by Gilbert (1995),

Table 1.6 Occurrence of *L. monocytogenes* in different food types on retail sale in England and Wales.

		L. monocytogenes isolated	
Year	Number samples analysed	Number	%
Cooked chicken			
1988/1989	527	63	12
1989	102	27	26.5
1991	169	46	27.2
1991	983	91	9.3
1993	119	6	5
Soft cheese			
1987	222	23	10.4
1988/1989	1135	67	5.9
1989/1990	131	0	0
1991/1992	251	10	4
1995	1437	16	1.1
Paté			
1989	155	46	29.7
1989	1698	162	9.5
1989	65	13	20
1990	626	25	4
1990	96	3	3.1
1991/1992	40	1	2.5
1994	3073	80	2.6

Source: Gilbert, (1995).

(Table 1.6) could also be a contributing factor (together with better Government advice to susceptible individuals [See 1.7]) in the lower incidence of listeriosis seen in pregnant women the United Kingdom after 1989 (McLauchlin and Newton, 1995). Also, in the case of paté (Table 1.6) the initial raised levels of *L. monocytogenes* were associated with imported paté from a single manufacturer causing outbreaks of human listeriosis in the UK (McLauchlin *et al.,* 1991), (Table 1.5).

1.7 EPIDEMIOLOGY

1.7.0 Human listeriosis

Although human listeriosis can be caused by all 13 serovars of *L. monocytogenes,* serovars 1/2a, 1/2b and 4b are responsible for the majority of the cases globally (Ryser and Marth, 1991). However there are some geographical differences, with 4b predominating in Europe and an even distribution of 1/2a, 1/2b and 4b in the USA and Canada (Farber and Peterkin, 1991). Epidemics of listeriosis have been documented [See1.6.5], however the disease can also occur sporadically (Filice *et al.,* 1978; Larsson, 1978; Simmons *et al.,* 1986, Hall *et al.,* 1995), (Table 1.7) and as a result the worldwide incidence of listeriosis is unknown. Given occasional mild symptoms, the non-detection of sporadic cases, and the non-requirement of statutory notification in most countries, the true figure of human listeriosis cases is likely to be far higher than the current data suggest (Gellin and Broome, 1989; Jones, 1990). Postulated figures for the true number of cases of listeriosis in the human population vary between 1 and 12 per population million, and though there is doubt as to the true number, it is known that there has been a dramatic increase in the amount of recorded cases in industrialised nations in the last twenty years (Jones, 1990).

An epidemiological study of human listeriosis infection in the United Kingdom in the last decade, indicate that for unknown reasons, human listeriosis is most

prevalent during the late-Summer, early-Autumn (McLauchlin *et al.,* 1990; McLauchlin and Newton 1995). The same authors also report that after 1987-1989, when incidence of human listeriosis peaked in the United Kingdom, due to consumption of contaminated paté from a single source (McLauchlin *et al.,* 1991), the overall incidence of listeriosis returned to close to the levels of the early 1980's. In the period after 1989 McLauchlin and Newton (1995) reported that listeriosis in pregnant women decreased significantly, due possibly to the elevated profile by the British Government of the dangers of eating potential risk foods, such as soft cheeses and paté whilst pregnant and if immunocompromised (Anon, 1989,a,b,c; Anon, 1991). In the same post-1989 period, McLauchlin and Newton (1995) indicated a slight increase in listeriosis in all other persons. This may be due to the changes within the population, with an increase in the average age and also an increase in the amount of immunocompromised individuals present.

Table 1.7 Examples of sporadic human listeriosis linked to food.

Food	Patient (age,gender)[a]	Health status
Fish	54Y F	NK[b]
Cooked chicken nuggets	52Y F	Lupus, steroids
Cheese	66Y M	Heart disease, diabetes, alcoholic
Alfalfa tablets	55Y M	Chronic hepatitis, steroids, antacids
Turkey frankfurter	61Y F	Cancer
Cheese	36Y F	Healthy
Whey cheese	40Y F	Healthy
Cook/chill chicken	31Y F	Pregnant
Vegetable rennet	29Y F	Pregnant
Human milk	24D F	Healthy
Homemade sausage	NK	NK
Salted mushrooms	80Y M	Healthy
Cajun meat and rice	>55Y	Healthy
Raw meat	76Y F	Chronic renal failure
Smoked cod roe	38Y F	Underlying disease
Ice cream, fresh cream	64Y M	Healthy
Pork sausage	43Y M	Healthy

[a] Y, Years; D, days; M, male; F, female; [b] NK, not known .

Adapted from Farber and Peterkin, (1991).

1.7.1 Animal listeriosis

Listeriosis in animals is noted with increasing frequency worldwide, though the exact incidence is unknown. Seasonal variation in the incidence of animal listeriosis have been noted, with peak periods between November to March, notably February and March (Gray and Killinger, 1966). A known reason for this is the consumption of contaminated silage during this period [See 1.6.4]. Changes in the production of silage in Holland have reduced the prevalence of listeriosis, linked to silage, in cattle (Dikjstra, 1987).

Listeria monocytogenes serovar 1/2a is responsible for most cases of animal listeriosis, as in humans, though serovar 4b has also been isolated (Ivanov, *et al.,* 1985; Wilesmith and Gitter, 1986). *Listeria ivanovii* serovar 5, though not a common cause of ovine listeriosis in the United Kingdom, has been shown to be prevalent abroad (Ivanov, 1975).

Of particular concern is the economic cost of animal listeriosis. Ryser and Marth (1991) noted that agricultural economies of Australia and Norway were effected by loss due to listeriosis of, respectively, one million and 200-2500 sheep during the 1970s. In the United Kingdom an outbreak of listeriosis in a housed flock of sheep causing the loss of 21 ewes and 88 lambs, cost the farmer £5000 (Low and Renton, 1985). Dijkstra, (1987) also reported that in The Netherlands between 1970 and 1985 there were between 234-928 cases of bovine listeric abortion annually, causing an average of 3.2% of total cattle abortion during that period. Another financial consideration, linked with human foodstuffs, is the prevention of contamination of the production process by irradicating contact with infected animals and faeces. This can be performed in several ways: improvement of animal housing and feed, and improved testing during manufacture, and also the effective bacteriological control of *Listeria*, by methods such as vaccination as was demonstrated in Norway during the 1980s (Gudding *et al.,* 1985; Gudding *et al.,* 1989). Vaccination using two attenuated strains of *L. monocytogenes*

decreased ovine listeriosis from 4 to 1.5%. In addition there was a marked decrease (1.1%-0.7%) in the amount of abortions seen in the vaccinated flocks (Gudding *et al.,* 1985; Gudding *et al.,* 1989).

1.8 METABOLISM

1.8.0 Substrates and metabolic pathways

Listeria monocytogenes is a facultative anaerobic organism with cell yields under anaerobiasis 80% of those when grown aerobically (Pine *et al.*, 1989). The metabolism of *L. monocytogenes* is poorly understood, with very little information available on metabolic pathways. Carbohydrate is essential for the growth of the *L. monocytogenes* however, Miller and Silverman (1959), reported the organism uses glucose as a carbon source, and is not capable of using xylose, gluconate, arabinose or ribose as an alternative carbon source. Pine *et al.,* (1989) demonstrated growth of several strains of *L. monocytogenes* on the following carbon sources: glucose, lactose, rhamnose and xylose (poor growth). Premaratne *et al.,* (1991) additionally showed that fructose, mannose, N-acetylglucosamine, N-acetylmuramic acid, glucosamine, cellobiose, trehalose, maltose and glycerol also served as adequate carbon sources in the absence of glucose, although growth using maltose and glycerol was weak.

Studies using resting cells indicate that catabolism of glucose is via the Embden-Meyerhof pathway, with lactic acid being the main product after anaerobic growth and pyruvate, acetoin, and lactic acid the main end products after aerobic growth (Miller and Silverman, 1959). Evidence for the pathway was provided by the presence of two key enzymes: 6-phosphogluconate dehydrogenase, and glucose-6-phosphate dehydrogenase (Miller and Silverman, 1959). Pine *et al.*, (1989) showed the extent of aeration was important in the relative amounts of end products produced, with vigorous aeration causing a reduction in the amount of lactic acid, and an increase in the amount of acetic acid. Increased aeration

also increased the cell yield, though in a defined medium the absence of adequate iron nullified this effect, (Trivett and Meyer, 1971). Detailed studies of metabolic end products of *L. monocytogenes* have noted differences, Daneshvar *et al.,* (1989) indicated that after aerobic fermentation using glucose as a carbon source, *Listeriae* produce acetic, isobutiric, butyric, iso-valeric, phenylacetic, lactic, 2-hydroxybutyric, 2-hydroxyvaleric, and 2-hydroxyisocaproic acids and also of the alcohol and amine derivatives produced, identified acetylmethylcarbinol, butylamine and putrescine. In a recent study of *L. monocytogenes*, Romick *et al.,* (1996) postulated that the presence of the large numbers of iso and isohydroxy acids seen in the Daneshvar *et al.*, (1989) study may have been due to oxidation of the amino acids present in the 'rich' medium used. Furthermore Romick *et al.,* (1996) using a defined medium with glucose as the sole carbon source, noted the following end products (% carbon): Aerobic growth; lactate (28%), acetate (23%), acetoin (26%) and carbon dioxide (25% (estimated)). Anaerobic growth; lactate (79%), acetate (2%), formate (5.4%), ethanol (7.8%), and carbon dioxide (2.3%).

In studies using a defined medium, Trivett and Meyer (1971) showed that exogenous citrate cannot be utilised by *L. monocytogenes* and pyruvate, acetate, isocitrate, α-ketoglutamate, succinate, fumarate, and malate were unable to either, support growth in the absence, nor promote growth in the presence of glucose. Also the absence of key enzymes of the citrate cycle (α-ketoglutarate oxidation system, succinate dehydrogenase, isocitrate lyase and malate synthase), led Trivett and Meyer (1971) to speculate that *L. monocytogenes* possessed a split citrate cycle, containing both oxidative and reductive portions, with a principally biosynthetic, rather than a energy-producing, role.

Recent publications have indicated the presence of carbon source uptake pathways in *L. monocytogenes.* Mitchell *et al.,* (1993) reported that *L. monocytogenes* possesses a phosphoenolpyruvate (PEP) dependent uptake

pathway for fructose. This transmembrane uptake system enables a sugar to be taken up, against the concentration gradient, in a monophosphorylated form, together with pyruvate. No evidence was seen by Mitchell *et al.,* (1993) for other sugars, such as glucose, using the PEP uptake system. However this was refuted by Christenson and Hutkins (1994), who demonstrated the presence of a PEP-glucose uptake system in *L. monocytogenes.* Christenson and Hutkins, (1994) also showed the presence of a glucose transport system requiring ATP, suggesting that a proton-motive force uptake system exists for glucose in *L. monocytogenes.*

1.8.1 Further nutritional requirements

A variety of other nutrients are required for the growth of *L. monocytogenes.* Publications relating to suitable defined media for the organism show differences in the amount, and type of vitamins and amino acids necessary or stimulatory for growth. In their review, Jones and Seeliger, (1990) indicated that biotin, riboflavin, and thioctic acid were essential vitamins, and that the amino acids cysteine, glutamine, isoleucine, leucine, and valine are required for growth of the organism in a defined medium. Jones and Seeliger, (1990) also reported that arginine, histidine, methionine, and tryptophan have a stimulatory effect on the growth of the organism. A portion of the work presented in this thesis should resolve some the uncertainty relating to the most suitable combination of vitamins and amino acids required for the growth of *L. monocytogenes.*

The presence of iron stimulates the growth of the organism in aerated and static cultures of the organism (Sword, 1966; Trivett and Meyer, 1971). The use of aeration, will increase cell yields and growth rate, but only in the presence of adequate levels of iron (Trivett and Meyer, 1971). Feresu and Jones (1988), also showed that adequate iron and aeration is required for the detection of cytochromes, and although all strains are catalase-positive, insufficient iron, low

levels of meat and yeast extract, and insufficient aeration may give a catalase-negative phenotype (Jones and Seeliger, 1990).

1.9 THE GROWTH OF *L. monocytogenes* AT LOW TEMPERATURES

Because of the association between listeriosis and food, the ability of *L. monocytogenes* to grow at low (refrigeration) temperatures is of concern to the food industry. It has been postulated that in at least two outbreaks of human listeriosis caused by the consumption of contaminated coleslaw (Schlech *et al.,* 1983), and milk (Fleming *et al.*, 1985), the refrigerated storage of the products and/or ingredients may have allowed the organism to multiply, in effectively a cold enrichment stage. The survival and growth of *L. monocytogenes* in a range of foodstuffs at low temperatures has been documented (e.g.. Hudson and Mott, 1993; Rosenow and Marth, 1991; Johnston *et al.,* 1986; Marth, 1986). Because of the undoubted ability of the organism to exhibit growth, often within the shelf life of a refrigerated foodstuff, awareness at a retail and consumer level, of preventing cross-contamination and maintenance of refrigerators at correct temperatures is of paramount importance.

There have been a number of studies concerning the psychrotrophic nature of *L. monocytogenes.* The ability of the organism to grow at very low temperatures is greatly affected by the type of medium employed. In demonstrating the growth of the organism at low temperatures (-0.5°C), Walker *et al.,* (1990), indicated that lowest value at which growth occurs may be unknown as they encountered difficulties with the medium freezing at temperatures below -0.5°C. Walker *et al.*, (1990) also demonstrated that a preincubation of the inoculum at 4°C reduced growth lag times at very low growth temperatures and also increased growth rate compared to cells grown at similar growth temperatures, but inoculated with cells grown at 30°C. Similar findings have been reported by Buchanan and Klawitter, (1991). These findings indicate than if physiological adaptation(s) required for

the growth of *L. monocytogenes* at low temperatures are made during a 'pre-incubation' period, such as in an ingredient, the dangers of high levels of contamination in refrigerated foodstuffs are increased. A situation similar to this scenario arose in an outbreak of human listeriosis in Canada when *L. monocytogenes* contaminated cabbage was cold stored for a number months before being added (raw) to the finished coleslaw, which was subsequently implicated in the outbreak (Schlech *et al.,* 1983).

Growth at the lower end of the temperature spectrum effects the ability of the organism to withstand pH extremes, George *et al.,* (1988), demonstrated that the minimum pH value at which growth was demonstrated was 4.39, 4.39, 4.62, 4.62, and 5.23 at growth temperatures of 30, 20, 10, 7, and 4°C, respectively. Comparable results were also seen by McClure *et al.,* (1989) and Farber *et al.,* (1989). In a recent study involving batch and continuous culture of *L. monocytogenes,* Patchett *et al.,* (1996) indicated that the growth temperature and the growth rate of cells is important in the ability of the organism to withstand environmental conditions. This study showed greater acid tolerance (pH 2.5) occurred with cells grown at 30°C compared to cells grown at 10°C at the same growth rate. Cells grown at 30°C tended to be more tolerant to acid stress when grown at low growth rates. No difference was seen between cells grown at 10°C and 30°C to a heat shock (55°C), irrespective of the growth rate. Patchett *et al.,* (1996) concluded that these findings may have implications for cold stored foods that use a reduction in pH to control bacteria such as *L. monocytogenes.*

The role of glycine-betaine and other compounds such as proline and carnitine in conferring osmotolerance in *L. monocytogenes,* when the organism is grown in the presence of high salt concentrations such as NaCl, is known (Patchett *et al.,* 1992, 1994; Beumer *et al.,* 1994). Glycine-betaine has cryoprotectant qualities also, and growth of *L. monocytogenes* at low temperatures is enhanced by its ability to take up increased amounts of glycine-betaine (Ko *et al.,* 1994; Beumer

et al., 1994). Ko *et al.,* (1994) indicated that the uptake system was rapidly activated after the cells were cold-shocked (i.e. cells were rapidly exposed to low temperature: 7°C), and similarly inactivated if subsequently exposed to higher temperatures (30°C). Also, when the temperature was raised to 30°C there was some evidence of efflux of glycine-betaine from the cells. Ko *et al.,* (1994) also indicated that uptake of glycine-betaine was not mediated by induction of a transport protein, and though any temperature induced changes within the membrane composition [See 10.1.3] may not account for influx of glycine-betaine into the cell following cold-shock, changes in membrane composition may account for the slight increase in glycine-betaine transport in cells grown under cold conditions (i.e. long term adaptation). Though the mechanism by which glycine betaine acts as a cryoprotectant is not understood, Ko *et al.,* (1994) postulated that they may be similar to those during osmotic stress. Such mechanisms are: to act as a stabilising agent (Yancey *et al.,* 1982) to prevent the aggregation, and maintain the solubility of cellular proteins or to alter the physical properties of the cell membrane (Rudolph *et al.,* 1986).

The effects of growth temperature on the virulence of *L. monocytogenes* is somewhat equivocal. Early work indicated that low culture temperatures enhanced the virulence of the organism (Gray and Killinger, 1966; Wood and Woodbine, 1979). In recent years, attempts have been made to assess the effect of temperature on the virulence of the organism, using a mouse model of disease. These studies, (Czuprynski, 1989; Stephens *et al.,* 1991) indicate that when the organism is grown at low temperatures (4°C), before being administered intravenously, to mice there is a significant effect on virulence. This effect was seen to be dose dependant with virulence becoming apparent only at doses above 10^4 viable Listeria (Stephens *et al.,* 1991). An increased virulence was not seen when cold incubated *L. monocytogenes* was administered orally to mice (Czuprynski, 1989; Stephens *et al.,* 1991). More recent work by Bucic and Avery (1996), however, has indicated that in mice, different strains of *L. monocytogenes*

generally showed a decrease in virulence when kept at 4°C in phosphate buffered saline (pH: 7.0 and 5.5), for a period of four weeks. Also, the production of listeriolysin O, an extracellular haemolysin and virulence factor present in all virulent strains of *L. monocytogenes* appears to be reduced at lower growth temperatures (26°C compared to 37°C), (Datta and Kotharay, 1993), though there are earlier reports of growth at low temperatures enhancing the production of listeriolysin O (Gray and Killinger, 1966; Wood and Woodbine, 1979).

1.10 MECHANISMS OF PSYCHROTOLERANCE IN MICRO-ORGANISMS

The adaptability of microorganisms to temperature extremes is well known (e.g. see reviews by Herbert, 1989; Russell, 1990; Hazel and Williams, 1990; Suutari and Laakso, 1994). Microorganisms, particularly spoilage and pathogenic organisms, that are able to survive and multiply at below recommended refrigeration temperatures are of particular concern to the food industry.

The accepted definitions for any microorganisms able to grow at the lowest end of the temperature scale are those of Morita, (1975). These propose that organisms deemed to be psychrophilic have an optimum growth temperature <15°C, and an upper temperature limit of <20°C. Psychrotrophic, or psychrotolerant bacteria, though capable of growing at or close to zero, have an optimum growth temperature >15°C. Compared with psychrotrophs, psychrophiles have a narrower growth range, and some have an optimum growth temperature as low as <10°C. In accordance with these criteria *L. monocytogenes,* with an optimum growth temperature of between 30 and 37°C (Seeliger and Jones, 1986), but also able to grow at temperatures below freezing (Walker *et al.,* (1990) can be deemed a psychrotrophic (psychrotolerant) bacterium.

In order to assess the mechanisms by which microorganisms are able to adapt to grow at low temperatures, primary consideration must be given to the role of bacterial membranes. Membranes play a central role in the mediation of cellular activity by acting as a barrier to electrolyte diffusion, whilst remaining as solvents for a variety of membrane constituents. In his 1989 review, Herbert noted that an alteration in membrane lipid composition in response to a variation in growth temperature is a principle response of microorganisms. By such mechanisms it is likely that the organism is able to regulate the activity of solute transport systems and the function of essential membrane-enzymes.

In assessing the ability of *L. monocytogenes* and other microorganisms to exhibit growth at low temperatures, temperature induced changes in membrane lipids and fatty acids, variation in cellular enzymes, and the role of cold induced (cold shock) proteins, as well the effect of temperature on substrate uptake will each be considered in greater detail below.

1.10.0 Bacterial lipids and fatty acids

Bacterial lipid composition varies between 2-20% dry weight. This value however, increases to 40-70% dry weight in the bacterial cell membrane bilayer, where lipid is the major component (Quinn, 1976, Lechevalier and Lechevalier, 1988). The most important property of a membrane lipid is that it is amphiphilic, i.e. it has both polar and non-polar regions and though a range of non-polar lipids (e.g. sterols and carotenoids) are also seen, it is vital for the membrane that a balance between the hydrophobic and hydrophilic regions of the lipids is sought (Russell, 1989). Natural membranes consist predominantly of phospholipids though other polar lipids (e.g. Phosphoglycolipids) are also present, in the form of an asymmetric bilayer. The polar outer regions (phosphate head group) are able to interact with the aqueous phase inside and outside the cell, whereas the hydrophilic interior of the bilayer consists of hydrophobic fatty acid acyl chains

stacked at right angles to the plane of the bilayer coupled by a terminal methyl group (Herbert, 1989; Russell, 1989, Suutari and Laakso, 1994).

Both regions of the polar lipid are able to interact with other compounds. For example peripheral and integral cellular proteins are able to bond electrostatically with the polar head groups of phospholipids, integral proteins are also able to interact hydrophobically with the acyl chains, facilitating a degree of control by the acyl region on processes such as active transport, and membrane-bound enzymes. (Russell, 1989; Suutari and Laakso, 1994). Also if the integral protein spans the membrane and interacts with other proteins it can have a membrane strengthening role (Russell, 1989). Russell, (1989) and Suutari and Laakso, (1994) also noted other mechanisms of interaction: carbohydrates can interact specifically with the polar region of the polar lipids (e.g. membrane derived oligosaccharides in the periplasm of the Gram negative microorganisms), also lipid-lipid interactions occur at both the polar and non-polar regions of the membrane lipid, thereby enabling co-operation between lipids during temperature induced phase transition [See1.10.3]

The bilayer is therefore able to directly control permeability of substances in and out of the cell, providing a selective barrier to potentially hazardous substances, participate in the regulation of the activity of membrane bound enzymes, thereby controlling biosynthetic processes such as the synthesis of macromolecules, cellular energy production and, photosynthetic processes (Suutari and Laakso, 1994).

Structurally, phospholipids are formed by acylating a glycerol backbone, containing two esterified fatty acid chains [See below] to a phosphoric acid molecule, (Lechevalier and Lechevalier, 1988; Suutari and Laakso, 1994). The simplest phospholipid is phosphatidic acid (1,2-diacyl-*sn*-glycero-3-phosphate), and esterfication by a variety of mono- and polyhydroxy- compounds give rise to

phosphodiesters of which the glycerophospholipids are seen commonly in bilayers (Ratledge and Wilkinson, 1988; Suutari and Laakso, 1994).

The (glycero)phospholipids are most common in lipid bilayers. Types seen are: phosphatidylinositol (PI), phosphatidlyglycerol (PG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine and diphosphatidylglycerol (DPG), (Lechevalier and Lechevalier, 1988). These are not present in all bacteria, however, and some are more common in certain bacterial groups than others. Greater amounts of PG are generally seen in Gram positive bacteria, as are, aminoacylphosphatidylglycerols, especially amongst organisms grown under acidic conditions; whilst PE is particularly common in Gram negative bacteria (Lechevalier and Lechevalier, 1988).

Free fatty acids do not accumulate freely intracellularly in their natural forms as they are toxic, but are linked to amines or alcohols, in particular glycerol, giving acylglycerols (Ratledge and Wilkinson, 1988). Bacteria contain a wide range of fatty acids ($CH_3(CH_2)_nCO_2H$), commonly between C_{10} and C_{20} in length, with C_{14}-C_{20} predominating (Ratledge and Wilkinson, 1988). Types seen are straight, branched chain or cyclopropane, and saturated or monounsaturated (normally ω-7 i.e.. the double bond is 7C from the terminal methyl group). Occasional dienoic fatty acids are present, and polyunsaturated fatty acids are seen rarely, and mainly restricted to *Mycobacterium* sp. (Asselineau *et al.,* 1972; Lechevalier and Lechevalier, 1988).

Branching of fatty acids is usually confined to the appearance of one or two methyl groups on the alkyl chain. Where the methyl group is at the penultimate (ω-1) carbon atom (distal from the carboxyl group on the fatty acid) the fatty acid is termed *iso-*. If the methyl group is on the third carbon atom (ω-2) fatty acid is termed *anteiso-*.

Long-chain branched fatty acids (mycolic acids) exist in some organisms such as *Rhodococcus* and the *Mycobacterium-Nocardia-Corynebacterium* bacterial group, and include other functional groups at the methyl end of the fatty acid molecule; these can be hydroxyl, oxo, epoxy, and cyclopropane, (Lechevalier and Lechevalier, 1988).

1.10.1 Lipid and fatty acid content of Gram negative bacteria

Though some differences occur in Gram negative bacterial lipids, members of Pseudomonads and other Gram negative eubacteria generally contain similar phospholipids: PE, PG, and DPG (Goldfine, 1982).

The major fatty acid is usually 16:0 with lesser amounts of 12:0, 14:0 and 18:0 whilst the major unsaturated fatty acids are 16:1(9) and 18:1(11), and also hydroxylated fatty acids are frequently reported (Lechevalier and Lechevalier, 1988; Suutari and Laakso, 1994). There are occasional reports of cyclo-propane fatty acids (Lechevalier, 1977), and polyunsaturated fatty acids (Roessel and Asseninaeu, 1980), however relatively few bacteria contain branched or odd-numbered fatty acids (Lechevalier and Lechevalier, 1988; Suutari and Laakso, 1994).

1.10.2 Lipid and fatty acid content of Gram positive bacteria

There are considerable differences in the polar lipid composition of Gram positive bacteria compared to Gram negative; PE, found commonly in Gram negative bacteria are found consistently in bacilli (Goldfine, 1972), and a number of actinomycetes only (Lechevalier *et al.,* 1977). In contrast PG, although present in Gram negative organisms, is found in much higher proportions in Gram positive organisms (Goldfine, 1972).

The fatty acid content of Gram positive bacteria differ considerably from Gram negative because, with a few exceptions (e.g. *Streptococcus, Pediococcus*)

(Lechevalier, 1977; Drucker and Lee, 1981), in many Gram positive bacteria (e.g. *Bacillus* spp., *Micrococcus* spp., *Staphylococcus* spp., *Kurthia* spp., *Corynebacterium* spp., and *Brevibacterium* spp.) (Kaneda, 1977; Kaneda, 1991; Komogata and Suzuki, 1987; Batrakov and Begelson, 1978; Goodfellow *et al.*, 1980; Bousfield *et al.*, 1983, Suutari and Laakso, 1994,) the presence of branched chain fatty acids (*iso-*, and *anteiso-*) are characteristic. In addition, as is the case for Gram negative bacteria, there are only occasional reports of polyunsaturation or cyclic fatty acids (Lechevalier and Lechevalier, 1988; Suutari and Laakso, 1994).

1.10.3 Alteration in membrane lipids in response to temperature changes

It has been known for a considerable amount of time that a primary response of microorganisms to a change in environmental temperature is an alteration in the composition of membrane lipids (e.g. Marr and, 1962; Russell, 1971; Oliver and Colwell,1973; Cullen *et al.*, 1971, Bhakoo and Herbert, 1979). In addition, it is well documented that these changes generally involve the fatty acid moieties, rather than polar region of polar lipids (e.g. Marr and Ingraham, 1962; Cullen *et al.*, 1971; Russell, 1971; Fukunaga and Russell, 1990; Suutari and Laakso, 1994). The maintenance of the integrity of the bacterial membrane at reduced growth temperatures is thought to occur principally by decreasing the melting point of a proportion of membrane fatty acids in order to facilitate normal functioning of the lipid bilayer. These changes in membrane fatty acids produce membranes with an overall lower gel to liquid-crystalline transition temperature (T_c), thereby enabling the liquid-crystalline state, characterised by thinner, loosely packed more 'fluid' membranes to be maintained at all growth temperatures. The mechanism of altering membrane fatty acids in response to changing growth temperatures in order to maintain constant fluidity levels is termed 'Homeoviscous adaptation' (Sinensky 1974) and this adaptation may permit the correct functioning of membrane functions such as solute transport (Overath *et al.*, 1970); the assembly of transport proteins (Tsukagoshi and Fox, 1973); and

the activity of membrane bound enzymes (Kimelberg and Papahadjopoulos, 1972).

Though the changes appear to primarily involve the fatty acid moieties, there have been reports of changes in the amount of polar lipids present, for example Bhakoo and Herbert, (1979) showed an increase in the total phospholipid content of a psychrophilic *Vibrio sp.* with a reduction in growth temperature. Also, despite the numerous reports of a reduction in environmental temperature eliciting a change in the membrane lipids of many microorganisms, there are several examples of a reduction in the temperature having little effect on the lipid profile, for example, Hunter and Rose, (1972) were unable to show any significant variation in the fatty acids of *Saccharomyces cerevisiae* when grown at 30°C and 15°C. Also Bhakoo and Herbert, (1980) were unable to detect any significant differences in either fatty acid or phospholipid composition in a number of psychrotrophic pseudomonads when grown at temperatures between 20 and 0°C. Thus, whilst homeoviscous adaptation occurs in some microorganisms it is not a universal response to growth at low temperatures. The T_c of a membrane is not sharply defined, and microorganisms have been shown to tolerate substantial levels of gel-phase lipid (e.g.. Jackson and Cronan, 1978; Melcoir, 1982).

A change in growth temperature can effect the degree of fatty acid unsaturation, chain length, branching and cyclisation within the bacterial membrane. (See Suutari and Laakso, 1994; Herbert, 1989; Russell, 1990). Chan *et al.,* (1971) compared the proportions of fatty acids in thermophilic, mesophilic and psychrophilic species of *Clostridia,* and indicated the amount of unsaturated fatty acids increased with decreasing growth temperatures (thermophiles (10%), mesophile (37%), psychrophile (52%)), the psychrophilic organism also showed significantly higher proportions of shorter chain fatty acids. Also Bhakoo and Herbert, (1979) looked at the effect of growth temperature on four psychrophilic

Vibrio spp. This study showed that the strains responded differently to a decrease in growth temperature with either an increase in the amount of unsaturated or short chain fatty acids present. Similarly *Micrococcus cryophilus* produces increased amounts of short chain fatty acids in response to a reduction in growth temperature (Russell, 1971), and Marr and Ingraham, (1962) showed that *E. coli* respond to a decrease in growth temperature by increasing the amount of unsaturated fatty acids present. The effect of these raised amounts of unsaturated and shorter chain fatty acids is a reduction in the gel liquid-crystalline transition temperature (Russell, 1989).

Variation in branched chain fatty acids tends to be restricted to Gram positive psychrophiles and psychrotrophs. Russell (1990), reported unpublished results for a psychrotrophic *Bacillus sp.* showing the major effect of a decrease in growth temperature was an increase in *anteiso* 15:0, relative to *anteiso* 17:0 with only minor changes in *iso / anteiso* ratios. Similar findings for *Bacillus subtilis* were seen by Kaneda (1977). *anteiso-* fatty acids tend to have lower phase transition temperatures than *iso-* or straight chain fatty acids of a similar size. A further response to reduction in growth temperature is a reduction in the amount of cyclic fatty acids (if present) (See Suutari and Laakso, 1994).

1.10.4 Studies analysing the membrane lipid and fatty acid content of *Listeria monocytogenes*

There have also been a number of studies analysing the membrane lipid and fatty acid content of *L. monocytogenes,* both at a single, and a range of growth temperatures. All the studies used a complex medium and all used cells grown under batch culture, except Carroll *et al.,* (1968) who also used cells grown in a chemostat at a single temperature only (37°C).

The polar lipid composition of *L. monocytogenes* is reported to comprise of PG, DPG, galactosyl-glucosyldiacylglycerol and an uncharacterised glyco-phospholipid (Kosaric and Carroll, 1971; Shaw 1974).

Although some strain differences have been reported in *L. monocytogenes* (Raines *et al.*, 1968; Ninet, *et al.*, 1992), generally, two fatty acids predominate: the branched forms of 15:0 and 17:0. The presence of large quantities of branched fatty acids is in keeping with many Gram positive organisms [See 1.10.2]. Of the studies that analysed the forms of branching (Carroll *et al.*, 1968; Feresu and Jones, 1988; Ninet, *et al.*, 1992; Puttman *et al.*, 1993; Annous *et al.*, 1995) the *anteiso-* forms, in particular *ai*-15:0, were much more prevalent than *iso-* forms. There are a number of other fatty acids present in much lower (and often trace) amounts. These are dependent upon strain and include straight chain fatty acids from 12:0 to 23:0 as well as branched 13:0, 14:0, 16:0, 18:1, 18:2, (Raines *et al.*, 1968; Carroll *et al.*, 1968; Tadayon & Carroll, 1971; Feresu and Jones, 1988; Ninet *et al.*, 1992; Puttman *et al.*, 1993; Annous *et al.*, 1995).

1.10.5 The effect of growth temperature on membrane lipids of *L. monocytogenes*

In keeping with temperature induced changes in Gram positive psychrophiles and psychrotrophs [See 1.10.3] the most striking affect of a reduction in temperature on the fatty acid composition of *L. monocytogenes* NCTC 7973 is a progressive decrease in the levels of *ai*-17:0 at temperatures below 30°C, accompanied by a small rise in *ai*-15:0 (Puttman *et al.*, 1993; Annous *et al.*, 1995). In these studies however, *L. monocytogenes* was grown on solid complex medium, (Puttman *et al.*, 1993), or in a complex broth (Annous *et al.*, 1995), and a degree of caution has to be applied before correlating these changes solely to growth temperature variation [See 1.11]. Neither of the studies (Puttman *et al.*, 1993; Annous *et al.*, 1995). showed any increase in fatty acid unsaturation and neither looked at any growth temperature induced effects on whole polar lipids.

1.10.6 The effect of a reduction in temperature on cell proteins

In comparison with the amount of published material assessing the response of membrane lipids to growth at low temperatures, there are relatively few publications studying responses by proteins to reduced temperature. Studies relating to thermal stability of proteins in microorganisms concentrate on the effects of high temperatures, however, Farrell and Rose (1967) postulated that cessation of growth of mesophiles at low temperatures may be caused by inactivation of solute uptake systems, possibly due to changes in the spatial organisation of the lipid bilayer preventing solutes interacting with respective carrier proteins, or by a lack of energy to support active transport mechanisms. It is also possible that enzyme systems in psychrophilic organisms need not be as efficient as mesophilic counterparts in view of their (relatively) slow growth rates (Russell, 1990). There is, as yet, no evidence supporting these hypotheses, but psychrotrophs must be able to generate energy at a different temperatures in order to demonstrate growth over a range of temperatures. This may occur either by possessing cold-tolerant enzyme systems (e.g.. lactate dehydrogenase in the psychrotroph *Bacillus psychrosaccharolyticus* has a optimum activity temperature lower than its mesophilic counterparts within the genus (Schlatter *et al.,* 1987)), or alternatively, a psychrotrophic organism may posses separate enzyme systems that work over different temperature ranges (e.g.. an obligately psychrophilic *Vibrio* (ABE-1), has two isocitrate dehydrogenase isoenzymes with different thermo stability properties, one that is deactivated above 20°C, and reactivated at temperatures approaching zero, and a second that is stable at temperatures above 20°C (Ochai *et al.,* 1979, 1984)).

Other responses of cellular proteins to a reduction in growth temperature have been documented for *Escherichia coli* (Herendeen *et al.*, 1979; Jones *et al.*, 1987; Goldstein *et al.*, 1990). Jones *et al.*, (1987) showed that when the growth temperature of an exponential culture of *E. coli* was abruptly decreased from

37°C to 10°C, growth stopped for several hours before continuing at a lower growth rate. During this lag in growth there was a considerable change in the cellular proteins, with a dramatic reduction in the amount of proteins produced, of these, thirteen were synthesised at raised levels compared with 37°C and the protein synthesised at the highest rate during this period was not detectable at 37°C (Jones *et al.,* 1987). Of the twelve remaining cold shock proteins, seven were identified as polynucleotide phosphorylase, Nus A, initiation factor 2α, initiation factor 2β, Rec A, dihydro-lipoamideacetyltransferase, and pyruvate dehydrogenase. Thus some of the proteins induced in the shift to low temperature include a set of proteins involved in transcription and translation (Nus A and initiation factor 2) and particularly mRNA degradation (polynucleotide phosphotransferase). This is of interest as a previous report had indicated that a cause of cold induced growth cessation is the blocking of an early stage of protein synthesis (Broeze *et al.,* 1978). Goldstein *et al.,* (1990) also analysed cold shock proteins in *E. coli,* and saw a similar cold induceable protein, to that of Jones *et al.,* (1987). Upon further analysis, the protein was shown to be a hydrophilic protein of 70 amino acid residues, which may be functionally homologous to an antifreeze polypeptide present in the fish, winter flounder (Yang *et al.,* 1988). Goldstein *et al.,* (1990) using cells grown at 37°C, with and without preincubation at 10°C, prior to freezing, showed up to a 70-fold increase in the survival if the 10°C preincubation step took place, indicating a possible antifreeze role for the cold shock protein. The presence of similar cold-shock proteins have been noted in *Bacillus subtilis* and *Streptomyces clavulgaris* (Willimsky *et* al., 1992; Av-Gay *et al.,* 1992). Francis *et al.,* (1995) indicated the presence of a protein sequence in *L. monocytogenes* homologous to a gene *cspB* that encodes the cold shock protein in *B. subtilis,* a stress induceable cold-shock protein that may protect *B. subtilis* from ice crystal formation during freezing (Willimsky *et al.,* 1992) Regulation of the gene in *L. monocytogenes* appears to be similar to *B. subtilis,* rather than in *E. coli* in that it is present at 30°C, but is more abundant at lower temperatures (10°C). There is no further information

concerning cold temperature induced changes in cellular proteins of *L. monocytogenes.*

1.10.7 Growth temperature effects on substrate uptake

The role of alteration in the lipid component in response to changes in growth temperature in order to maintain the integrity of the bacterial membrane has already been discussed. Several workers have indicated that the failure to maintain membrane integrity, results in substrate uptake being severely inhibited, which has a catastrophic effect on the ability of mesophiles to grow at low temperatures (Ingraham and Bailey, 1959; Rose and Evison, 1965; Morita and Buck, 1974). There are conflicting findings for substrate uptake in psychrophiles and psychrotrophs, Russell (1971) showed that lysine uptake occurred at similar levels at 20°C and 0°C in *Micrococcus cryophilus*. Also, Baxter and Gibbins, (1962) showed that sugar transport in psychrophilic *Candida* sp. occurred independently of growth temperature and these findings were supported by the work of Herbert and Bell (1977). They demonstrated that in a psychrophilic *Vibrio* the assay temperature and not the growth temperature was the principle factor controlling the rate of sugar uptake.

In a detailed study on the effect of reduced temperature on psychrophiles and psychrotrophs, Bhakoo and Herbert (1979, 1980) indicated that in each of the psychrophiles studied, maximum glucose and lactose uptake occurred at 0°C, a lower than optimal growth temperature, but a temperature at which maximum cell yield was attained. An increase in the growth temperature brought about a rapid decline in uptake rates (Bhakoo and Herbert, 1979). Conversely, the psychrotrophic organisms studied showed maximal uptake occurred at closer to 20°C with a rapid decline in uptake with decreasing temperature (Bhakoo and Herbert, 1980). Similarly, Paul and Morita, (1971) showed a reduction in uptake of ^{14}C glutamate by a psychrotrophic *Vibrio* sp. at low temperatures. Also Fukunaga and Russell, (1990) studied uptake rates using two Gram negative

psychrotrophic species, one with more psychrophilic properties. This study supported the findings of Bhakoo and Herbert, (1979, 1980) as a reduction in growth temperature favoured glucose uptake in the 'psychrophilic' organism, and an increase in the temperature favoured uptake in the 'psychrotrophic' organism. Fukunaga and Russell, (1990) postulated that psychrophiles and psychrotrophs from a permanently cold environment may be more suited to growth at low temperatures by their ability to alter their membrane lipid fatty acid composition, and as a consequence, their ability to take up nutrients.

There has been a limited amount of work concerning glucose uptake in *L. monocytogenes* at low temperatures. The ability of the organism to take up a non-metaboliseable analogue of glucose, 2-deoxy-D-glucose under batch conditions at a range of temperatures between 3 and 45°C was studied by Wilkins *et al.*, (1972). This work indicated that there was an increase in both the rate and capacity of 2-deoxy-D-glucose uptake by cells growing at low temperatures. Wilkins *et al.*, (1972) postulated that a cold-resistant transport system was present in *L. monocytogenes* that stimulated growth at low temperatures by providing high concentrations of intracellular substrates.

1.11 THE IMPORTANCE OF CULTURE CONDITIONS AND GROWTH MEDIUM COMPOSITION FOR TEMPERATURE STUDIES IN BACTERIA

Much published work looking at effects of growth temperature on bacteria have been performed using cells grown under batch conditions and/or using a complex medium. However, both of these factors are potentially problematic and may contribute to misleading results. This is true for all comparative studies of cells grown at a range of temperatures.

1.11.0 Medium composition

Complex medium may contain components such as yeast extract, plant extracts, and peptones that contain lipids that can be acquired by bacteria ,thereby giving rise to potentially misleading results (Ratledge and Wilkinson, 1988). A chemically defined medium containing no lipids, or components containing lipids can be used irradicate this problem. Additionally a defined medium with a single carbohydrate source permits detailed uptake analyses to be performed.

1.11.1 Culture conditions

When studying the effect of different growth temperatures on microorganisms it is vital to ensure that strict control is maintained over any potentially variable parameters. Such parameters include pH, nutrient concentration, dissolved oxygen, and, in particular, growth rate. Each can be tightly controlled only if the bacterial cells are continuously cultured using a chemostat. If continuous culture techniques are used for growth temperature studies for bacteria, any adaptations made by the microorganism can be directly and solely related growth temperature change. There have been several studies showing the effect of different culture conditions on composition of bacteria. For example Harwood and Russell, (1984) showed that as a proportion of total phospholipid present, phosphatidylglycerol increased to a maximum during the exponential phase of growth in *Micrococcus lysodeiktikus*. Similarly Oliver and Colwell, (1973) directly correlated changes in the proportions of phospholipids present in a marine *Vibrio* sp. to different phases of growth and Marr and Ingraham (1962) compared cells of *Escherichia coli* grown at different temperatures under batch and continuous culture and showed that there were higher amounts of saturated fatty acids present in the continuously cultured cells.

The results of each of these studies reinforce the requirement for continuously cultured cells to be used in all growth temperature studies. Therefore *L. monocytogenes* cells grown in defined medium using a chemostat were used for

the work described in this thesis, with temperature as the single variable and growth rate kept constant throughout. This enabled an analysis to be made of polar lipid and fatty acid composition, and also of differences in substrate uptake in *L. monocytogenes* at a range of temperatures between 30°C and 10°C. Although 10°C is at the upper end of refrigeration temperatures, it was chosen as it falls into the category of a psychrophilic temperature and permits the growth of *L. monocytogenes* in a chemostat with a more practical growth rate.

2. MATERIALS AND METHODS

2.1 BACTERIA

Three strains of *L. monocytogenes* were used, NCTC 4885 serovar 4b, NCTC 5105 serovar 3a and NCTC 7973 serovar 1a.

The strains were obtained from the culture collection, Department of Microbiology and Immunology, Leicester University and a stock kept at -70°C in vials containing sterile glass beads and tryptose soya broth (Oxoid) with 1.5% (v/v) glycerol (Jones *et al.*, 1983).

All bacteria were grown and maintained on tryptose soy agar (Oxoid). After overnight growth at 37°C all plates were stored at 4°C. All cultures were subcultured at least fortnightly, and always the day before required.

2.2 PREPARATION OF THE DEFINED MEDIA

Six previously published chemically defined media for *L. monocytogenes* were compared under batch conditions using 30°C static and shaking water baths. The media compared were those of Friedman and Roessler, (1961); Welshimer, (1963); Trivett and Meyer (1971); Ralovich *et al.*, (1977); Siddiqi and Khan, (1989); and Premaratne *et al.*, (1991).

Nitrilotriacetic acid and all vitamins, amino acids and bases were purchased from Sigma, and all other chemicals were from Fisons (AR Grade). Each constituent of the media was purchased specifically for this purpose, and removed from departmental circulation to ensure no contamination from other users.

All glassware was washed thoroughly with Decon 90, rinsed five times in tap water and then five times in double deionised water to remove any residue left

after the normal departmental glasswashing process. Double deionised water was used as the base for all of the defined media.

All vitamins, amino acids and bases in the media were made up as x10, x20, x50, or x100 concentrates, dependant upon solubility, after vigorous stirring and gentle heating. These solutions were sterile filtered (using Sartorius 0.2μm Midisart 2000 filtration units) and used as required in the media. The carbon source (glucose) was made up freshly as a concentrate and sterile filtered into the media. All other components of the media were sterilised by autoclaving at 120°C for 20 minutes at 20psi.

2.2.0 Composition of the defined media

All media were prepared as described in the respective publications, except that the agar was omitted from the medium of Ralovich *et al.*, (1977). Amounts shown are for 1 litre of medium.

(i) Friedman & Roessler (1961):

Salts A

NaCl	2.5g
$MgSO_4.7H_2O$	800mg
Na.Citrate	400mg
$MnSO_4.H_2O$	100mg

Salts B

K_2HPO_4	6g
Glucose	6g

Vitamins

Riboflavin	5mg

Thiamine·HCl	5mg
Biotin	5mg
Thioctic acid	5mg

Amino Acids

DL Alanine	800mg
DL Aspartic acid	800mg
L Glutamic acid	800mg
L Arginine·HCl	200mg
L Lysine·HCl	200mg
L Histadine·HCl	100mg
DL Isoleucine	200mg
L Leucine	200mg
L Phenyl alanine	100mg
L Proline	100mg
L Threonine	100mg
L Tyrosine	100mg
DL Valine	200mg
DL Tryptophan	200mg
L Cysteine·HCl	100mg
DL Serine	200mg
Glycine	100mg
L Glutamine	600mg
L Asparagine	500mg
Alanine	800mg
DL Methionine	200mg

(ii) Welshimer (1963):

Salts

KH_2PO_4	3.28g
Na_2PO_4	8.2g
$MgSO_4 \cdot 7H_2O$	409mg
Glucose	10g

Amino Acids

L Cysteine·HCl	100mg
L Leucine	100mg
DL Isoleucine	200mg
DL Valine	200mg
L Glutamine	600mg
DL Methionine	200mg
L Histidine·HCl	200mg
L Arginine·HCl	200mg
DL Tryptophan	200mg

Vitamins

Riboflavin	1mg
Biotin	100µg
Thiamine·HCl	1mg
DL Thioctic acid	10µg

(iii) Trivett & Meyer (1971):

Salts A

K_2HPO_4	8.5g
$NaH_2PO_4 \cdot 2H_2O$	1.7g
NH_4Cl	500mg

Salts B

Nitrilotriacetic acid	480mg
NaOH	240mg

Salts C

$FeCl_3$	29mg
Glucose	2g

Salts D

$MgSO_4 \cdot 7H_2O$	410mg

Amino Acids

L Cysteine·HCl	100mg
L Leucine	100mg
DL Isoleucine	200mg
DL Valine	200mg
DL Methionine	200mg
L Arginine·HCl	200mg
L Arginine·HCl	200mg
L Histidine·HCl	200mg

Vitamins

Riboflavin	1mg

Thiamine·HCl	1mg
D Biotin	100µg
Thioctic acid	10µg

(iv) Ralovich *et al.*, (1977):

Salts

K_2HPO_4	9.8g
KH_2PO_4	4.23g
Na citrate·$2H_2O$	470mg
$(NH_4)_2SO_4$	940mg
Na Thioglycollate	1.03g
$FeSO_4·7H_2O$	916mg
$MgSO_4·7H_2O$	188.3mg
$CaCl_2·6H_2O$	12.5mg
Glucose	9.41g

Amino Acids

DL Isoleucine	190mg
L Leucine	90mg
DL Valine	190mg
L Cysteine·HCl	380mg

Vitamins

Riboflavin	4mg
D Biotin	4mg
Thiamine·HCl	9.2mg

(v) Siddiqi & Khan (1989)

Salts

$(NH_4)_2SO_4$	5g
NaCl	5g
KH_2PO_4	1.5g
Na_2HPO_4	2.5g
KCl	50mg
$FeCl_3$	10mg
Glucose	4g

Amino Acids

DL Cystine	500mg
DL Isoleucine	500mg
DL Lycine	500mg
L Leucine	500mg
DL Methionine	500mg
L Phenylalanine	500mg
L Threonine	500mg
DL Tryptophan	500mg
DL Valine	500mg

Bases

Adenine	5mg
Cytosine	5mg
Guanine	5mg
Thymine	5mg

Vitamins

Riboflavin	5mg

Folic Acid	5mg
D Biotin	5mg
Thiamine·HCl	5mg

(vi) Premaratne *et al.*, (1991):

Salts A

Na_2HPO_4	6.4g
KH_2HPO_4	6.56g

Salts B

$MgSO_4 \cdot 7H_2O$	410mg
$MgSO_4 \cdot 7H_2O$	410mg
Fe citrate	88mg
Glucose	10g

Amino Acids

L Leucine	100mg
L Isoleucine	100mg
L Arginine	100mg
L Methionine	100mg
L Valine	100mg
L Cysteine	100mg
L Glutamine	100mg

Vitamins

Biotin	500µg
Riboflavin	500µg
Thiamine	1mg
Thioctic Acid	5µg

2.2.1 Preparation of inoculum

A loopful of *L. monocytogenes* was removed from a fresh TSA plate, plated onto a Welshimer medium (solidified using 1.5% w/v agar) and incubated at 30°C. Welshimer medium was chosen because it is the least complex of the defined media. After 36 hour growth on solid Welshimer medium, one loopful of cells was inoculated into 20ml of each of the chemically defined media and incubated at 30°C for 24-48 hours.

Growth on Welshimer solid medium and subsequently in small volumes of the respective media was performed to minimise any carry over of constituents from the TSA culture medium.

Growth in each of the defined media was initiated by removing 500µl of culture from the 20ml cultures and inoculating into 100ml of the same medium in 500ml bottles. Incubation took place at 30°C in a water bath, either statically or shaking (150RPM in a New Brunswick Gyratory water bath).

2.2.2 Measurement of growth

Growth in batch cultures was determined from the optical density at 600nm with a Perkin Elmer 550s spectrophotometer and by wet weight.

Growth rate constants (μ_{max}) were compared by a comparison of μ_{max} values. These values were calculated using the formula described by Herbert & Bell, (1977):

$$\mu_{max} = 2.303 \times \frac{\log Z - \log Y}{T_2 - T_1}$$

Where Z and Y are OD_{600} values at times T_2 & T_1 respectively

Values for wet weight were obtained by centrifuging cells from 20ml of culture in late stationary phase of growth in preweighed, capped bottles for 20 min at 3275 *g*. The supernatant was discarded and the bottles containing pelleted cells inverted over layers of tissue paper for 30 minutes before reweighing. Wet weight was expressed as g ml^{-1} of medium. Each experiment was performed at least in duplicate and the majority were done four times.

2.2.3 Growth under anaerobic conditions

The medium of Trivett and Meyer, (1971) was also used to investigate the growth of strain NCTC 7973 under anaerobic conditions. For this, 100ml of freshly prepared medium in a 100ml bottle was allowed to stand in an anaerobic cabinet at 37°C ('Compact' model, Don Whitely Scientific Ltd, Shipley, Yorkshire, UK) for a period of two to three hours, before inoculation in the same way as described before and subsequent incubation for a period of 24-36 hours. The atmosphere in the cabinet was 10% v/v carbon dioxide, 10% v/v hydrogen and 80% v/v oxygen-free nitrogen.

2.3 THE PREPARATION OF THE CHEMOSTAT, AND THE GROWTH OF *L. monocytogenes* IN CONTINUOUS CULTURE

Listeria monocytogenes strain NCTC 7973 was grown in a chemostat using the medium of Trivett and Meyer, (1971), [See 2.2.0].

2.3.0 Chemostat

The chemostat used in all the studies was a fully instrumented 1.0 litre Biolab minifermenter (FT Scientific Instruments, Bredon, Tewkesbury, UK.). For these studies a working volume of 500ml was used. All tubing used was of silicone and obtained from Altech (Hants. UK).

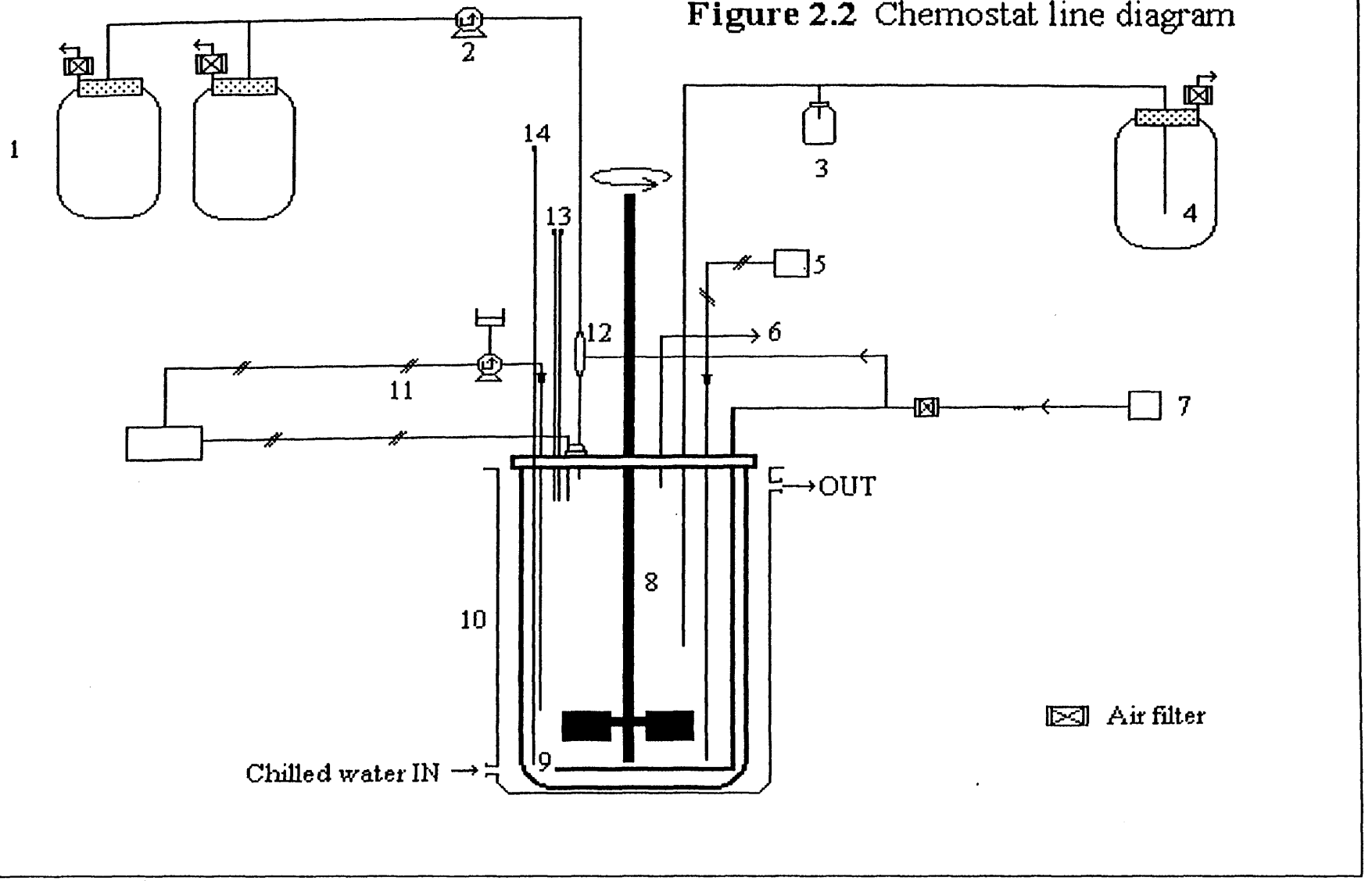
The use of a fermenter facilitated the accurate control of all parameters. The pH was automatically controlled so that the pH of the vessel culture was never outside set limits of 6.8-7.4. Any deviation from these values was prevented by the automated addition of 0.5M NaOH, or 0.5M HCl. The pH meter used also enabled the accurate monitoring of pH over a range of temperatures. Drifts in pH readings were adjusted by standardising the culture pH using an external laboratory pH meter every second day.

Dissolved oxygen was measured using a dissolved oxygen meter and probe (Type 2 Uniprobe, Uniprobe Ltd, Cardiff, Wales) and kept constant throughout the temperature range studied, by varying the percentage dissolved oxygen in relation to maximum dissolved oxygen values for specific temperatures. Any modifications to the dissolved oxygen concentration could be achieved by changing the impeller speed, or, for larger changes, by altering the flow rate of sterile air entering the vessel at the sparger using an adjustable valve.

The control of the vessel working temperature was achieved in two ways. For temperatures above that of ambient laboratory temperatures, a vessel heating element, coupled to a controller, was sufficient to keep temperatures to within +/- 0.2°C. For temperatures below ambient, a system specially designed for this study was used. This comprised of a glass cooling jacket around the vessel, through which was circulated chilled water at a temperature approximately 1°C below that required. The circulating water was chilled by means of a dip cooler in a water bath. 'Fine tuning' of the temperature to within +/- 0.5°C was performed by setting the vessel heating element to the desired temperature. This method, kept tight controls on the temperature range despite often large changes in the surrounding ambient temperature.

Figure 2.2 shows a line diagram of the chemostat system employed.

Figure 2.2 Chemostat line diagram

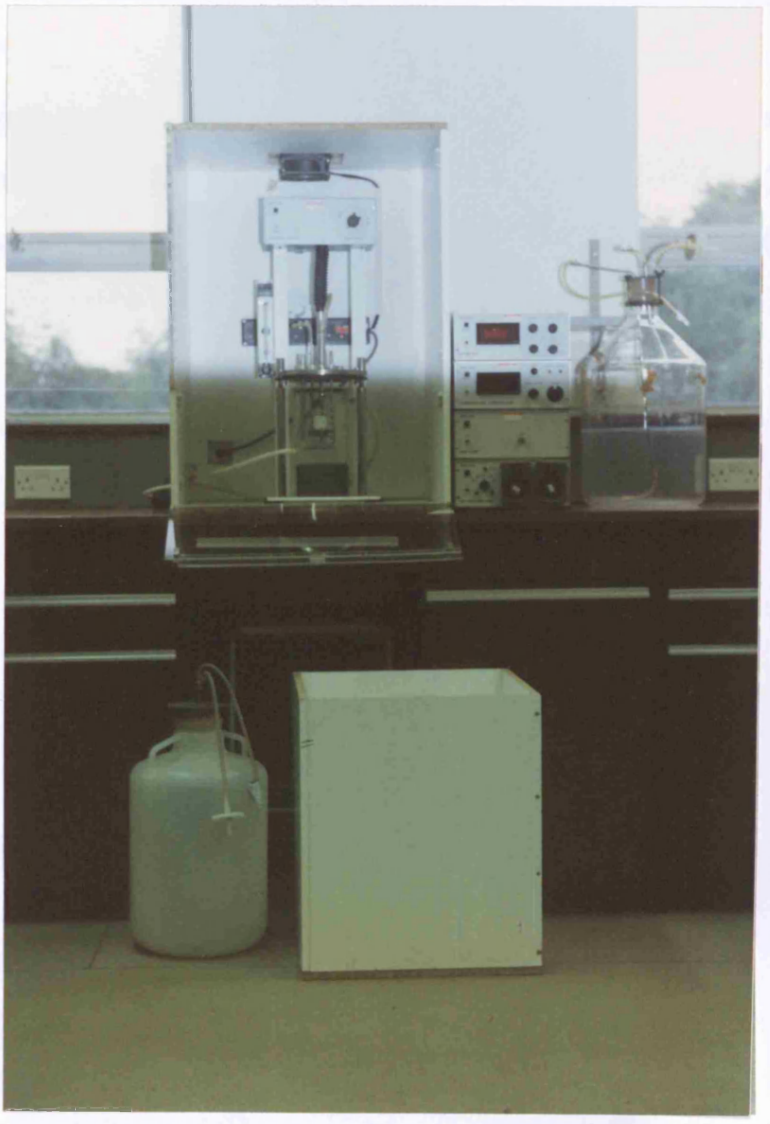


Key to Figure 2.2

1. 20 litre aspirators each containing 10 litres of sterile medium.
2. Peristaltic pump.
3. Sample port.
4. 20 litre harvest aspirator containing waste culture.
5. Dissolved oxygen control unit (probe and meter).
6. Air outlet.
7. Air pump.
8. Impeller.
9. Sparger.
10. Glass fermenter vessel (1 litre), surrounded by a glass cooling jacket.
11. pH control unit (pH meter, probe, and acid / alkali addition pumps).
12. Medium 'break tube'(Solomons, 1969).
13. Antifoam and inoculation ports.
14. Harvest port.

The chemostat was located in a small, purpose built safety chamber, consisting of a perspex fronted wooden box, which was maintained at a slight negative pressure by means of a small electrically driven fan fitted with a fibrous filter on the discharge side (Figure 2.3).

Figure 2.3 Safety chamber containing the chemostat



Continuous culture was obtained by pumping fresh medium in at a constant, known flow rate, and removing culture at the same rate. In the system employed here, culture was removed by restricting the flow of air from the vessel, thereby creating a positive pressure within. When the volume of culture inside the vessel exceeded the working volume (500mls) it was immediately forced out, through a portion of stainless steel tubing placed immediately above the medium.

The flow of fresh medium was controlled by the use of a peristaltic pump (LKB, Varioperpex, Sweden). Steady state was deemed to have been attained once five volumes of medium had been pumped into the vessel (i.e. 2.5 litres) (Levenspeil, 1962). The growth rate (μ) was calculated using the formula below (Pirt, 1975):

$$D=F/V$$

Where: D= Dilution Rate (h^{-1})
F= Flow Rate
V= working volume of the vessel

Under continuous culture conditions $\mu=D$

The flow rate into the chemostat vessel was calculated using an 'in line' glass pipette. Medium flow from the aspirator was temporarily diverted into the pipette and the flow rate (ml min^{-1}) was calculated with the aid of a stopwatch.

2.3.1 Sterilisation of the chemostat vessel

Sterilisation of the chemostat vessel, including both the pH and dissolved oxygen probes *in situ,* was performed by autoclaving in a B & T 225e autoclave. This autoclave was used as it had a slow exhaust phase which prevented electrolyte loss from the probes. 500ml of salts solution of the chosen defined medium was placed in the vessel and the fermenter head plate screwed down loosely. The waste, inoculation, fresh medium, pH (i.e. NaOH, and HCl), and antifoam [See 2.3.4] lines were all fitted with stainless steel connectors ('Stericonnectors' FT Applikon), one end of which was capped with a small piece of cotton wool covered in aluminium foil prior to sterilisation. The bottles and flasks containing the relevant solutions and the inoculum medium were all autoclaved separately, with the other end of the connector covered in aluminium foil. After autoclaving, the head plate was screwed down tightly and the air was immediately supplied to maintain a positive pressure within the vessel and prevent contamination. All the line connectors mentioned above were joined aseptically using a Bunsen flame. All sterile air filters used in the chemostat system were Midisart 2000 (Sartorius) and sterilised along with the vessel or the aspirators, (Figure 2.2). Spent culture was collected in an autoclavable 20 litre plastic aspirator.

2.3.2 The sterilisation of defined medium TM (Trivett and Meyer, 1971) for growth in continuous culture

Two 20 litre glass aspirators were used to supply the fermenter vessel with fresh medium. The top of each aspirator consisted of a large silicone stopper, clamped firmly into place by a wing nut and bolt, attached to a metal sleeve on the aspirator neck. Three stainless steel pipes entered the aspirator via the stopper, the largest of which was long enough to facilitate removal of all the medium, as required. The other two pipes were smaller and were used to enable air to enter the aspirator, via a sterile filter, and a second to enable entry of the heat labile components of the medium.

Sterilisation of the aspirators were carried out by autoclave (Intermed, Dent & Helliyer) with the autoclave temperature probe placed in a bucket containing 10 litres of distilled water, and running the autoclave at a sterilisation program of 30 minutes at 135°C. The volume of salts solution present in the aspirators (9.85 litres) was within the sterilisation capability of the autoclave.

In order to minimise any possibility of contamination during aspirator change-over the two aspirators were autoclaved joined together in series with a single connection to the vessel system, thereby facilitating the addition of 20 litres of medium. A separate connector between the aspirators allowed another 10 litres to be added before the 'run' had to be terminated. Occasional contamination occurred during the connection of aspirators to the vessel, but this was minimised by immersing the connector ends in 70% (v/v) industrial methylated spirits and flaming. Latex gloves washed in Cetab, (1% (w/v),in 75% (v/v) ethanol) were also worn throughout the process.

A concentrate of the heat labile constituents of the medium was added to each of the aspirators. This concentrate was 150ml in volume and contained all the amino acids, vitamins and also glucose required in the TM medium. The concentrate was sterile filtered using a 'GS Stericup' (Millipore) self-contained filtration unit, the sterile solution was then poured into a sterilised 250ml glass bottle, and joined to each of the aspirators by carefully flaming and pushing a rubber stopper onto the bottle. This stopper was connected to the aspirator by silicone tubing via two 'in line' sterilised 'Swinnex' 47mm 0.2μm filters, all of which was covered in aluminium foil and sterilised along with the aspirators. After connection, as much as possible of the 500ml salt solution present in the vessel during autoclaving was pumped out, using the harvest line [See 2.3.5]. Fresh medium was then pumped rapidly into the vessel until the full 500ml of TM was present. At this time a vessel temperature of 30°C was set. 30°C was used as an initial temperature at all times to ensure rapid bacterial growth after inoculation.

2.3.3 Chemostat inoculum and inoculation of the chemostat

The chemostat inoculum was prepared in a conical flask with a side arm containing 100ml of TM medium. The side arm was used to inoculate the chemostat vessel via a length of silicon tubing and a stainless steel connector. Particular care was employed in the adding of the heat labile constituents of the medium as this was a prime source of contamination, therefore they were all sterile filtered into the flask by means of a 20ml syringe, small 0.2μm filter and an 18 gauge needle. Before inserting the needle the flask's foil-covered rubber stopper was thoroughly flamed. The inoculum was incubated overnight in a 30°C incubator before the connector was connected as previously described [See 2.3.1]. The inoculum was added into the vessel by means of gravity, the sterile vessel air-line being switched off to facilitate this.

2.3.4 Growth in continuous culture

After inoculation the impeller was switched on, setting '2' on the impeller speed controller was used as it gave good mixing of the culture without creating large amounts of foaming. If undue foaming was observed, a few drops of Antifoam A (Sigma, Poole, UK) was added, by gravity, via the sterile antifoam line. This proved sufficient to inhibit foaming for a period of about a week. The antifoam was silicone rather than oil based, thus preventing potential problems when analyses on the cellular fatty acid and polar lipid composition were performed. After the impeller was switched on for about one minute, the air line was switched back on and the vessel volume equibrilated to 500ml immediately. The aeration rate used initially was 0.8 volumes of air per volume of medium per minute, though there were small variations in this as a consequence of temperature variation.

A sample was removed via a small sample port present on the waste line (Figure 2.2) every 24 hours. 100μl of this sample was plated out in duplicate on tryptose

soy agar, incubated overnight at 30°C or 18°C (for chemostat temperatures of 20°C or below) and a Gram stain and examination using the 'Henry Technique' (Henry,1933) [See 1.3.0], performed on resulting colonies to check culture purity. OD_{600nm} readings were taken using samples taken in the same way twice a day and the results noted.

2.3.5 Harvesting of continuously cultured cells

After continuous culture at various temperatures was obtained in the chemostat, the cells were removed, via the harvest line, into sterile 150ml bottles. About 475ml of cells could be rapidly removed using this system and after removal of the culture the vessel was rapidly replenished by pumping in fresh medium. Typically approximately 400ml of culture was removed before the cells were harvested by centrifuging at 8500 *g* for 30 minutes in a Sorvall (RE-5B) centrifuge The cells were then washed twice using double deionised waster and centrifuged as before. The pelleted cells were then either used immediately or stored at -20°C until required.

2.4 FATTY ACID AND POLAR LIPID PROFILES OF *L.monocytogenes*

2.4.0 Extraction

Polar lipids were extracted by a modification of the method of Bligh & Dyer, (1959) as described by Kates, (1972).

The frozen cells were lyophilised using an Edwards 'Modulo' lyophiliser and 90mg placed in a 20ml bottle with a Teflon-lined steel cap. To this was added 2.75ml aqueous methanol containing NaCl (10ml 0.3% (w/v) NaCl : 100ml methanol).This suspension was stirred for 1 min using three or four metal staples as stirring bars. The suspension was then heated at 100°C for 10 minutes, allowed to cool to room temperature and then 0.75ml of 0.3% (w/v) aqueous NaCl and 1.25ml of chloroform (AR) were added. The mixture was shaken vigorously to

produce one phase, and then stirred for 3 hours at room temperature. The extracted cells were removed by centrifugation (3000rpm for 10 minutes) and the supernatant transferred to another, clean glass universal.

The sedimented cells were resuspended in 2ml chloroform : methanol : 0.3% (w/v) aqueous NaCl (50:100:40), stirred for 15 minutes, centrifuged as before and the supernatant pooled with that of the previous extract. To the combined extracts 1.75ml 0.3% (w/v) aqueous NaCl and 1.75 ml chloroform was added and all then centrifuged as before to aid phase separation.
The upper aqueous layer was removed carefully with a Pasteur pipette and discarded. The lower layer was evaporated to dryness under a stream of oxygen-free nitrogen gas.

2.4.1 Analysis of the fatty acid profiles

The cellular fatty acids of *L. monocytogenes* NCTC 7973 were analysed using Gas-Liquid Chromatography (GLC). Prior to analysis, the fatty acids were methylated in order to increase the volatility of the sample in the GLC column, thereby enabling the sample separation to proceed at lower temperatures. Methanolysis was performed in the first instance by the method of Minnikin *et al.*, (1975), described in the thesis of Feresu (1980). However this method gave poor yields of fatty acid methyl esters (FAMES) and was replaced by the method of Berger & Miller (1985) following communication with M.D.Collins,(AFRC Institute of Food Research, Reading, UK).

2.4.2 Preparation of FAMES (Berger and Miller, 1985)

In all cases the methylation proceeded using cells grown under continuous culture conditions as described above, with temperature being the single variable parameter.

50mg (wet weight) of cells were used for methylation in all cases. After weighing, the cells were placed in 15ml glass tubes with plastic, Teflon-lined screw caps. Methylation was started by the addition of 1ml of the saponification reagent (45g NaOH, dissolved in 150ml of methanol and 150ml of double deionised water), followed by 5 min incubation in a boiling water bath. It was important to ensure that the screw caps were tightly closed to prevent evaporation. After incubation, the tubes were removed, and vigorously vortexed for 30 seconds before being returned to the boiling water bath to complete a 30 min incubation.

After cooling, 2ml of the methylation reagent, (325ml 6.0M HCl, 275ml of methanol), was added to the tubes before incubating for 10 +/-1 min at 80 +/-1°C, this step is critical in both duration and temperature. This stage of the procedure drops the pH below 1.5 and facilitated the methylation of the sample fatty acids. The tubes were then cooled before 1.25ml of the extraction reagent was added (equal volumes of hexane and anhydrous ethyl ether), the tubes were then gently mixed by placing on a rotating platform for about 10 minutes. This reagent extracted the FAMES from aqueous to an organic phase is required for the GLC. Finally 3ml of basic wash (10.8g of NaOH dissolved in 900ml of double distilled water), was added and the contents mixed on the rotator for 5-15 minutes. This procedure cleaned the FAMES sample and reduced any contamination of the injector, column and detector of the GLC. After mixing, about 2/3 of the organic phase was carefully removed using a glass pipette and placed into a glass vial with a foil lined, plastic screw cap, and stored at -20°C until required.

2.4.3 Analysis of FAMES using GLC

The FAMES samples were analysed using GLC and were identified by comparison of retention times with known standards. Generally a mixture of bacterial FAME standards, (Matraya, Inc. Pleasant Gap, PA, USA) as used.

However for the identification of one fatty acid (*ai*-17:0) a single standard, (Matraya, Inc.) was used.

Two different GLC systems were used in the study, both of which differed in the column matrix used by Berger & Miller, (1985). Initially a Pye Unicam GCD chromatogram, with a scientific Glass Engineering FFAP Megabore column (code 12A05/BP210.5) was used. However problems were experienced with this system, the most important being that, despite extensive variation of the running conditions, it was incapable of resolving all 26 peaks in the mix of bacterial FAME standards, and as a result made identification of the form of branching present on the fatty acids impossible.

As a result, a second system at the Department of Chemical Engineering, Loughborough University of Technology, Loughborough, Leic, UK was employed. The GLC used here was a Pye Unicam series 304 GLC, fitted with a splitter, (ratio 50:1) and a 25m x 0.33mm id SGE Bonded Phase BP1 Column. Helium was used as the carrier gas ($20cm^3min^{-1}$). The temperature program used ramped from 100°C in 4°C min^{-1} increments to 250°C, after an initial holding at 100°C for 4 minutes. When the temperature reached 250°C it was held for the duration of the analysis. Detection was by flame ionisation. 1µl of the FAME sample was injected and the same amount of the FAME standards, using a glass syringe (Hamilton). The results were analysed using a Pye Unicam PU4810 Computing Integrator

2.4.4 Analysis of polar lipid profiles

Polar lipid analysis was performed on *L.monocytogenes* NCTC 7973 grown under the continuous culture conditions described for fatty acids. Two dimensional thin layer chromatography (TLC) was used to qualitatively analyse the polar lipids.

2.4.5 Separation of polar lipids

The polar lipids were separated by two dimensional TLC using commercially prepared 10cm x 10cm aluminium foil backed silica plates (Merck). Approximately 0.5ml chloroform : methanol (2:1) was added to the dried extract, and spotted onto the TLC plates using a drawn out Pasteur pipette, until the edge of the spot just became uneven (Unevenness indicates the beginning of overloading). The spots were allowed to dry for at least 1 hour and run using chloroform : methanol : water (65 : 25 : 4), as the first solvent system and chloroform : methanol : glacial acetic acid : water (80 : 12 : 15 : 4) as the second solvent system. The plates were allowed to dry for at least 1 hour (usually 4 or 5 hours) between the two dimensions.

2.4.6 Identification of the polar lipids

Primary identification

Five different reagent systems were used to reveal the identity of the polar lipids. A single reagent was used on every TLC plate, thereby necessitating a series of plates for each temperature. All the reagents were sprayed onto the plates by means of an air-gun (Jet Pak Spray Gun, Aerosols International Ltd) and have been used previously for studies of *Listeria denitrificans* (*Jonesia denitrificans*), Collins *et al.,* (1983).

Reagents:

Dodecamolybdophosphoric acid (Ross *et al.,* 1981).

10% (w/v) dodecamolybdophosphoric acid (Fisons) freshly prepared in ethanol was used as a non-specific reagent for the detection of all polar lipids present. The plates were heavily sprayed with the reagent, left to dry at room temperature in a fume hood for five minutes, before baking at 150°C for 10-15 minutes, during which time the plates were checked at intervals to prevent excessive darkening of the plates due to over-baking. After baking the polar lipids appeared as black spots on a green / yellow background.

Ninhydrin (Consden and Gordon, 1948).

Commercially prepared 0.2% ninhydrin in water saturated butan-1-ol (Fisons) was used to detect the presence of lipids containing free amino groups. The plates were liberally sprayed with the reagent, left to dry at room temperature in a fume hood for five minutes, and then baked for 5-10 minutes. For reasons of safety the oven was placed in the fume hood whilst the ninhydrin sprayed plates were baked. The amino containing lipids appeared as deep red/violet spots on a pale violet background.

α-Naphthol (Jacin and Mishkin, 1965).

This reagent was used to detect lipids containing sugar moieties and was prepared by adding 10.5ml of a mixture containing 15% (w/v) α-naphthol in 95% (v/v) ethanol, to 6.5ml of concentrated sulphuric acid. To this solution was added 40.5% (v/v) ethanol and 4ml water. The plates were evenly sprayed with this reagent until light brown and then baked at 120°C for 10 minutes. The polar lipids-containing sugar moieties appeared as purple / blue spots on a light purple background. Extreme care was taken in the identification of the true glycolipid spots as the presence of concentrated sulphuric acid in the reagent caused charring of other polar lipids in the sample, though these appeared as a dark brown colour, and were ignored.

Zinzadze (Dittmer and Lester, 1964)

Commercially prepared Molybdenum blue (Sigma) diluted 1:1 with 4.2M sulphuric acid was used as this reagent, and was used to detect the presence of phosphate-containing lipids. The plates were lightly sprayed with the reagent and left at room temperature. The phospholipids appeared after 5-10 minutes as light blue spots. Occasionally the plates required repeated light spraying with the reagent before the spots appeared and the presence of liquid sulphuric acid on the plates caused the rapid loss of blue colour, and appearance of a brown colour, and also a rapid degeneration of the plates as a whole. As a result of this, the need for rapid photographing of the plates was essential.

Secondary identification

In order to confirm the primary identification of the lipids, where possible polar lipid standards were run in the same solvent system on separate plates. Though commercially prepared standards were not available in all cases, they enabled presumptive identification of phosphatidyglycerol and diphosphatidylglycerol.

2.5 CARBOHYDRATE UPTAKE STUDIES

2.5.0 Investigation of the uptake and utilisation of different carbohydrates by *L. monocytogenes*

The ability of *L. monocytogenes* to utilise different carbohydrates at two different temperatures (10 and 30°C) was investigated under batch conditions using TM medium with glucose replaced by a variety of carbon sources. These experiments were undertaken as a prelude to investigating how temperature influences the ability of different sugars to support growth of *L. monocytogenes.*

The utilisation studies were carried out using 15ml of TM medium in glass bottles prepared in the same way as for the defined medium comparison [See

2.2], except glucose was replaced by other carbon sources to give a final concentration of 0.2% (w/v). The inoculum was also prepared in the same way as for the medium comparison [See 2.2.1]. Any possibility of carry-over of carbon source was checked using an inoculated bottle containing TM medium with no added sugar and looking for no growth. Growth of *L. monocytogenes* at this stage would indicate carbon source carry-over. Initially, to enable a large number of carbohydrates to be screened relatively rapidly, growth was judged by eye, on a no growth, poor growth, good growth basis. Any sugar supporting a degree of growth at either temperature, was retested once, before a fuller investigation and quantification of growth rate (μ_{max}) was performed.

Table 2.1 Carbohydrates used in utilisation experiments:

Carbohydrate
α-L-Rhamnose
D-Fructose
D-Maltose
D-Cellobiose
D-Mannitol
D-Mannose
α-methyl-D-Glucoside
D-Lactose
D-Galactose
D-Ribose
D-Arabinose
Dextrin
D-Glycogen
Sucrose
D-Sorbitol
α-methyl-D-Mannoside
D-Sorbose
α-D-Melibiose
α+Xylose
D-Glucose
No carbohydrate

All carbohydrates were of AR grade (or equivalent) and purchased from Sigma, Fisons or BDH.

2.5.1 Use of membrane vesicles

Carbohydrate uptake studies were carried out initially using membrane vesicles. Preparation of the vesicles was based on the method of Kaback (1971), and involved a two step preparation. Initially, converting the bacteria to a osmotically sensitive form (protoplast), followed by a controlled osmotic lysis in a hypotonic medium in the presence of nucleases and a chelating agent. Cells were stored at the appropriate growth temperature prior to use. Also all enzyme incubations, and centrifugation steps were performed at the same temperatures to that of growth, in order to minimise any alteration of membrane characteristics caused by exposure to a change of temperature during the experiment. As a result of this it was occasionally necessary to increase the incubation length or the amount of enzyme present in some of the reactions.

Harvest

The cells used in the preparation of the membrane vesicles were removed directly from the chemostat and centrifuged at 16000 *g* for 20mins in a Sorval (RE-5B) centrifuge until the supernatant was clear. The cells were then washed twice in 10mM TRIS-HCl pH 8.0 (at appropriate growth temperature) before being resuspended (1g wet weight per 80ml) in 30mM TRIS-HCl pH 8.0 containing 20% (w/v) sucrose and gently mixed using a magnetic stirrer.

Protoplast formation

Potassium-EDTA pH 7.0 and lysozyme (lyophilised, Sigma) were added (10mM and 0.5mg/ml respectively). incubated for 30 minutes (or longer) at the relevant temperature.

Preparation of vesicles

The cells were centrifuged at 16000 *g* until the supernatant was clear (usually 20-30 mins), The resultant pellet was then resuspended in the smallest possible volume of 0.1 M potassium phosphate buffer, pH 6.6, containing 20% (w/v)

sucrose, and 20mM magnesium sulphate, before being thoroughly homogenised using a Teflon and glass hand homogeniser. Prior to homogenisation, DNase and RNase were added at a concentration of (3-5mg/ml) so that their total volume in the lysate (see below) was approximately 10µl/ml.

After homogenisation the concentrated homogenate was poured directly into 300-500 volumes of 50mM potassium phosphate buffer, pH 6.6 pre-chilled or heated to the relevant temperature and vigorously swirled. This was repeated a further two times, using the same buffer containing potassium-EDTA pH 7 at a final concentration of 10mM and then $MgSO_4$ (final concentration 15mM) were added.

Isolation of vesicles

At this point the membrane vesicles had been formed and as a result there was no longer a need to treat the preparations at different temperatures. The membrane vesicles were isolated as follows: The lysate was centrifuged at 16000 *g* for 30 minutes (or until the supernatant was clear) before the pellet was resuspended (again following homogenisation using a Teflon and glass hand homogeniser) in 0.1M potassium phosphate buffer pH 6.6 containing 10mM EDTA at 0°C.

Removal of cell debris

The removal of the vast majority of whole and partially lysed cells was by a low speed centrifugation of the vesicle suspension (800 *g* for 30 minutes), before the supernatant was carefully removed and centrifuged at 37000 *g* for 30 minutes. This procedure was repeated at least twice, and until there was less than 1 bacterium per field using ^{x}1000 phase contrast microscopy.

Washing of isolated vesicles

The final vesicle pellet was washed twice by resuspension followed by homogenisation in 0.1M potassium phosphate pH 6.6 containing 10mM EDTA and centrifugation at 36900 *g* for 30 minutes.

Density centrifugation

To remove any remaining whole and partially lysed cells a final purification step, involving a sucrose density centrifugation was performed on the vesicle preparation.

This method was modified from that of Kaback, (1971) and Buckley and Hamilton, (1994); and involved the use of a discontinuous gradient of 20% and 60% (w/v) sucrose in 0.1M potassium phosphate buffer pH 6.6 containing 10mM $MgSO_4$. Initially the vesicles were suspended in the 20% (w/v) sucrose solution and vigorously homogenised initially using a plastic, then glass Pasteur pipette, followed by a hand homogeniser. 3ml of this solution was then overlaid over 10 ml of 60% (w/v) sucrose solution in 17ml Polyallomer tubes. Centrifugation took place overnight at 64000 *g* in a swing bucket-type rotor using a Sorvall OTD-50B ultracentrifuge. After centrifugation the vesicles were carefully removed from the interface between the two different sucrose concentrations then washed twice in 0.1M potassium phosphate pH 6.6 containing 10mM EDTA followed by centrifuging at 37000 *g*.

Storage

After the last washing stage, the vesicles were resuspended in 0.5-1ml of 0.1M potassium phosphate buffer pH 6.6 at a concentration of 5-10mg dry weight (4-7 mg protein per ml). The vesicle preparations were then stored at -70°C until required for uptake studies

Quantitation

In order to quantify the amount of vesicles prepared, a protein assay was performed (BCA, Pierce USA), and the purity of the sample, was gauged by a comparison of initial CFU/ml with final CFU/ml. The final value for viable cells should not exceed 0.05% of the viable cells from which they were obtained (Kaback,1970).

2.5.2 Use of whole cells

The uptake of carbohydrates using intact *L. monocytogenes* cells was also studied. The method used was a modified version of Herbert & Bell,(1977) and Wilkins *et al.,* (1972) and in order to measure the rate of glucose uptake by the cells a radiolabelled, non-metaboliseable analogue, 2-deoxy-D-[U-^{14}C]-glucose was used. The use of this compound, gave values for total uptake and uptake rate that would be comparable to glucose, without giving rise to any problems relating to metabolism of the carbon source.

The cells used were taken from the chemostat when continuous culture was attained at three temperatures, 10, 20, and 30°C. The cells were harvested by centrifugation, at 16000 *g* in a Sorvall (RE-5B) until the supernatant was clear (20-30min). In order to maintain cell viability the cells were not frozen, but used immediately. The cells were then resuspended in 150mM TRIS-HCl, pH 7.2 at the appropriate growth temperature to an OD of 1.0 (+/- 0.05) and a dry weight of 0.8mg ml^{-1}.

Efforts were made to ensure that the cells remained at their chemostat growth temperature. 1ml volumes of cell suspension were added to a similar volume of reaction buffer containing 75mM TRIS-HCl, pH 7.2, 1mM 2-deoxy-D-glucose (Sigma) and 0.4μCi 2-deoxy-D-[U-^{14}C]-glucose (Amersham) (specific activity 299mCi/mmol). The reaction was then incubated at three separate temperatures:

10, 20, and 30°C, and 0.5ml aliquots of the reaction removed for counting at time intervals of 15 secs and 30, 60 and 120 minutes.

500µl was removed from the reaction mix at the required times, the cells were then isolated using a Buchner filtration assembly and 22mm, 0.45µm cellulose nitrate filter membranes. The cells were washed *in situ* using 20ml of ice cold deionised water

After air drying for 2-3 hours the filter membranes were placed in counting vials containing 4ml of Optiphase (LKB Scintillation products), and radioactivity was measured using a scintillation counter (Minaxi Tricarb 4000). Background readings were calculated using vials containing the scintillant only Any adhesion of the 2-deoxy-D-glucose to the membranes was measured by repeating the experiment in the absence of cells. 2-Deoxy-D-glucose uptake values were calculated as the mean of three 5min counts and there were five replicates at each incubation temperature. Statistical analysis was performed using SPSS computer statistics package.

3. RESULTS

3.1 DEFINED MEDIA

3.1.0 Comparison of the six defined media for growth of *L. monocytogenes*

All the chemically defined media supported growth of all the test strains of *L. monocytogenes* at 30°C when inoculated with an overnight culture grown on Welshimer medium. However no increase in optical density was detected upon second transfer to the medium of Siddiqi and Khan (1989), despite three attempts with each of the strains. This inability of the medium to sustain sequential growth has been noted previously (Premaratne *et al.,* 1991), and consequently it was not investigated further.

Table 3.1 shows the ability of the five remaining media to support the sequential growth of three strains of the organism in shaking culture, and Table 3.2 shows the ability of the media to support the growth of *L. monocytogenes* NCTC 7973, at 30°C statically. The results shown in Table 3.1 indicate that, in shaking culture, each of the five media were able to support the sequential growth of the three *L. monocytogenes* strains examined, except the medium of Welshimer, (1963) which was unable to support the sequential growth of strain NCTC 5105. In addition, Table 3.1 also shows that the highest overall values for maximal growth rate (μ_{max}) and yield (dry weight, mg ml^{-1}) were provided by the FR and TM media (Friedman and Roessler (1961), and Trivett and Meyer, (1971)). Also, studies with a single strain grown statically at 30°C, indicate that the same two media (TM and FR) gave the highest yield and the second highest value for μ_{max} (Table 3.2).

These results provided the basis for deciding the suitability of the media, however as the chosen medium was to be used for continuous culture studies, where large quantities of sterile medium are required, the requirements for their production had to be considered. Of particular importance in producing the medium was a concentrate containing sufficient heat labile components in a small enough

volume to permit aseptic filtration into the autoclaved sterilised media salt solution. On this basis, the medium of Trivett and Meyer, (1971), was chosen as the defined medium for all chemostat studies not only because it sustained good growth with each of the strains tested under both static and shaking conditions, but also the small number of heat labile constituents (glucose and 11 amino acids or vitamins), permitted the production of a manageable volumes of a sterile concentrate. By comparison, the medium of Friedman and Roessler (1961), had 26 heat labile constituents (glucose and 25 vitamins or amino acids). Thus production of a manageable volume of sterile concentrate containing these constituents would be relatively difficult. Also this medium (FR), produced a precipitate when the salt solution was autoclaved, and though this was noted by the authors, as was its disappearance when the concentration was reduced ten-fold, this reduction of the salt concentration resulted in a medium unable to support any of the strains tested here.

Table 3.1 Maximal growth rates (μ_{max}) and yields of *L. monocytogenes* strains cultured with shaking in five different defined media at 30°C.

	Media									
	TM		P		R		FR		W	
Strains	μ_{max}	Yield	μ_{max}	Yield	μ_{max}	Yield	μ_{max}	Yield	μ_{max}	Yield
L. monocytogenes NCTC 7973	0.31	4.10	0.10	2.24	0.20	1.18	0.22	6.07	0.26	5.11
L. monocytogenes NCTC 4885	0.25	3.06	0.21	3.79	0.07	0.70	0.04	3.00	0.08	2.70
L. monocytogenes NCTC 5105	0.16	1.55	0.04	1.4	0.03	0.71	0.23	3.05	NG	NG

TM, Trivett and Meyer, (1971); P, Premaratne *et al.,* (1991); FR, Friedman and Roessler, (1961); W, Welshimer, (1963); NG, no growth on second transfer. Yield (wet weight, mg ml^{-1}); μ_{max} (h^{-1});. Data shown are the mean of two to four experiments.

Table 3.2 Maximal growth rates (μ_{max}) and yields of *L. monocytogenes* strains cultured without shaking in five different defined media at 30°C.

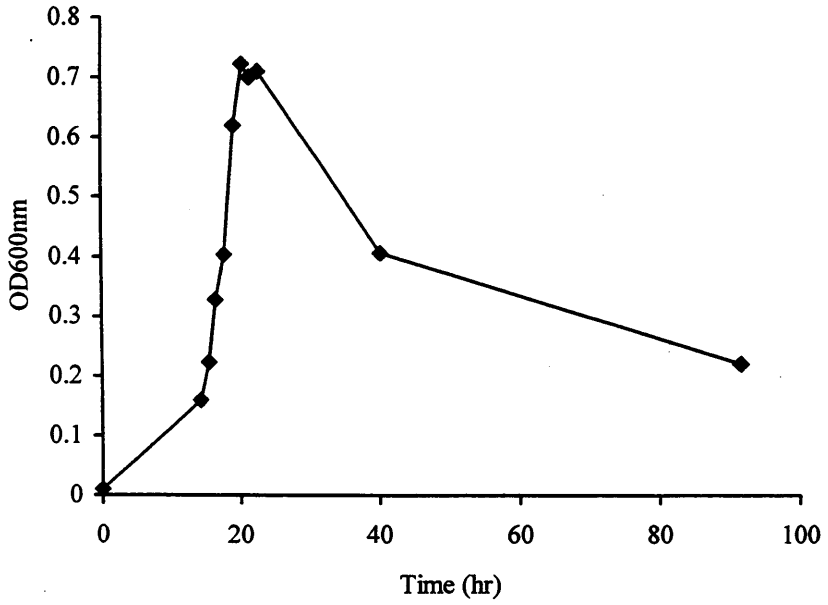
	Media									
	TM		P		R		FR		W	
Strains	μ_{max}	Yield	μ_{max}	Yield	μ_{max}	Yield	μ_{max}	Yield	μ_{max}	Yield
L. monocytogenes NCTC 7973	0.23	2.60	0.14	2.23	0.20	1.19	0.16	3.89	0.24	3.08

TM, Trivett and Meyer, (1971); P, Premaratne *et al.,* (1991); FR, Friedman and Roessler, (1961); W, Welshimer, (1963); μ_{max} (h^{-1}); Yield (wet weight, mg ml^{-1}). Data shown are the mean of two to four experiments.

3.1.1 Anaerobic batch culture of *L. monocytogenes* NCTC 7973

The ability of TM to support growth of *L. monocytogenes* NCTC 7973 anaerobically was examined. Static growth at 37°C in an anaerobic cabinet showed a similar growth rate (0.27 h^{-1}) to that of the 30°C aerobic static study, but a rapid cell lysis, noticeable by a rapid reduction in OD_{600nm} values, was seen at the end of the exponential growth phase (Figure 3.1). The source of the lysis was not studied in detail, but some characterisation was done: lysis did not occur if the same strain was grown aerobically at 37°C using TM medium (data not shown). Also after lysis had occurred, both the medium supernatant and the residual pellet were checked for the presence of phage by J. McLauchlin, PHLS, Colindale, London, UK, and none were found. Therefore it is possible to conclude that the repeated lysis is likely to be a facet of the anaerobic culture.

Figure 3.1 Cell lysis of cells grown anaerobically at 37°C using TM medium.



Data shown are the mean of four separate experiments.

3.2 CULTIVATION OF *L. monocytogenes* NCTC 7973 IN A CHEMOSTAT

3.2.0 The sterilisation of the defined medium

As described in the defined media comparison [3.10], the medium of Trivett and Meyer (1971) (TM) was chosen for the chemostat and therefore all subsequent studies. From the onset, there were two main logistical problems regarding its use that had to be overcome. Initial problems concerned the satisfactory autoclaving of the complete salts solution in a large enough quantities to keep the changing of spent medium aspirators to a minimum. This changeover process was a common cause of contamination in continuous culture. Also further, and more common sterilisation problems were encountered during the sterile filtering of the heat labile components of the medium into the aspirators after autoclave sterilisation of the salts solution.

Initial studies concerning the sterilisation of the salts solution showed that the autoclave employed (Intermed, Dent & Helliyer), sterilised the medium consistently only if the volume contained in the aspirators was reduced from 20 to 10 litres. Therefore, for all continuous culture studies, the volume of medium contained each of the aspirators was 10 litres (9.85 litres salt solution and 150ml of heat labile concentrate added after autoclaving).

The majority of the problems of chemostat vessel contamination were experienced whilst trying to achieve the sterile addition of the 150ml concentrate of heat labile medium constituents, described in the Materials and Methods section [See 2.3.2]. Despite the fact that use of repeated 0.2μm sterile filtration steps in this process tended towards over-caution, it provided an adequate way of adding the concentrated solution without contamination, and in view of the amount of time and inconvenience experienced due to aspirator (and therefore chemostat) contamination, it was deemed necessary.

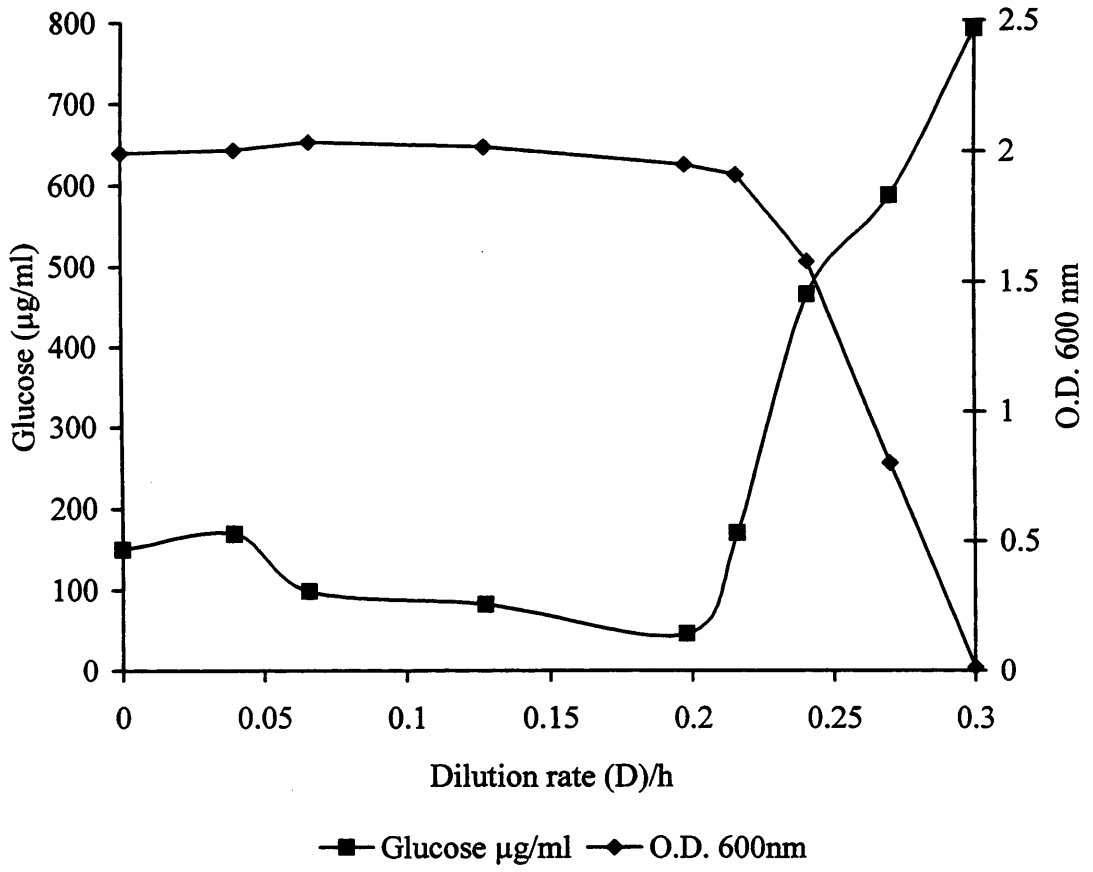
3.2.1 Continuous culture of *L. monocytogenes* NCTC 7973 in a chemostat

Initial studies concerned the growth of *L.monocytogenes* in continuous culture at 30°C in TM medium under glucose limiting conditions. Residual glucose levels in the vessel were assessed using the method of Dubois *et al.*, (1956).

As indicated in the materials and methods [See 2.3.0] under steady state conditions, the cell concentration in the vessel remains constant (i.e. formation of new biomass equals the loss of cells from the vessel), and the growth rate (μ) is controlled by the dilution rate (D) such that μ=D. The effect of increasing the flow rate does not significantly effect the concentration of the growth limiting nutrient until D approaches μ_{max}. This point is termed the critical dilution rate (D_c) and is manifested by a drop in cell concentration, and an increase in the residual concentration of the growth limiting substrate (Figures 3.2 and 3.3). D_c values for *L. monocytogenes* at 30°C and 10°C were estimated from Figures 3.2 and 3.3 as $0.22h^{-1}$ and $0.025h^{-1}$ respectively.

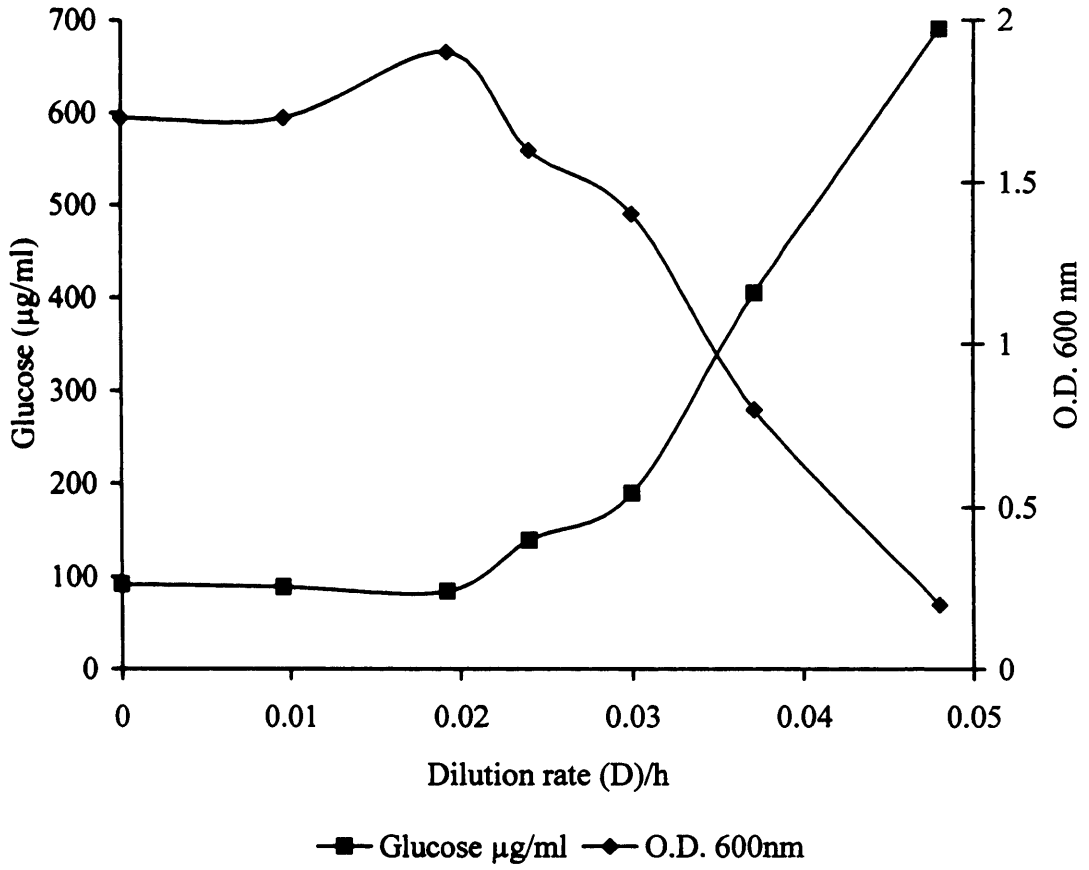
After these experiments were performed, cells were subsequently grown under carbon limitation and a growth rate of $0.02h^{-1}$, a value slightly lower than the D_c at 10°C was selected as the growth rate for cells at all growth temperatures.

Figure 3.2 The effect of dilution rate on the growth of *L. monocytogenes* NCTC 7973 in a chemostat at 30°C.



Cells grown in TM medium (Trivett and Meyer, 1971) under carbon limitation; residual glucose assayed by method of Dubois *et al.*, (1956)

Figure 3.3 The effect of dilution rate on the growth of *L. monocytogenes* NCTC 7973 in a chemostat at 10°C.



Cells grown in TM medium (Trivett and Meyer, 1971) under carbon limitation; residual glucose assayed by method of Dubois *et al.*, (1956)

3.2.2 Cell lysis during continuous culture

Almost from the onset of the use of a chemostat to culture *L. monocytogenes* cell lysis was observed. The problem occurred in two forms, the first of which occurred soon after the inoculation of the chemostat, at 30°C when the culture was growing under batch conditions (Figure 3.4).

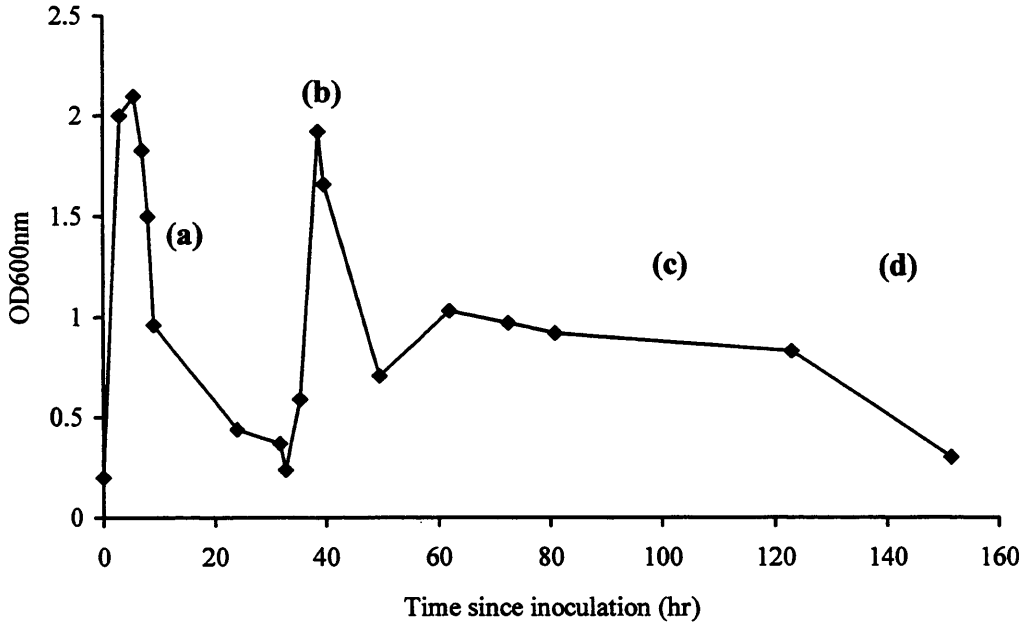
(a). (See Figure 3.4) After the addition of relatively large inoculum (20% of the final working volume) to the chemostat, the cells grew rapidly to an optical density (OD_{600nm}) of approximately 2.0. There then followed an equally rapid drop in OD_{600nm} indicating the occurrence of cell lysis. This problem was tentatively linked to the exhaustion of a key nutrient within the medium, and though it is not possible to identify the nutrient from the available data, a small amount of culture was removed at 32h and an assay (Dubois *et al.,* 1956), indicated very low amounts of glucose present (180μg/ml).

(b). (See Figure 3.4) Rapid addition of fresh medium (40% of working volume) after 32 hours caused the cells to grow back.

(c). (See Figure 3.4) After the increase in cell mass described in (b), medium continued to be added to the vessel but at a lower, though arbitrary flow rate (35ml h^{-1}) This value was chosen in order to obtain continuous culture of the cells with a growth rate significantly less than the previously calculated D_c value (Figure 3.2). The fluctuation seen in the time period (39-62 hours) is possibly due to a transient response by the culture in response to changes in the medium addition rate. By 72 hours the culture had adjusted to the changes in the medium flow rate and was adjusting to steady state.

(d). (See Figure 3.4) The reduction in OD_{600nm} seen here is due to washout, rather than cell lysis. It appears that for reasons unknown cell growth ceases at 123 hours, and the decline in OD_{600nm} is compatible with cells being washed out of the vessel (exponential decay).

Figure 3.4 Typical cell lysis of *L. monocytogenes* NCTC 7973 immediately after inoculation of the chemostat.



Conditions (See text for full description)

Cells grown under carbon limitation at 30°C.
(a) Zero dilution rate as medium pump not switched on as building up cell mass.
(b) Rapid addition of fresh medium after 32h preceded by cell lysis.
(c) and **(d)** Medium flow rate reduced to 35ml h^{-1}.

Figure 3.4 indicates that, despite the ultimate re-occurrence of cell lysis there may be a measure of protection to be had if the pump was switched on before any lysis had occurred. As a result of this, in subsequent experiments the medium pump was switched on immediately after inoculation of the vessel, and removal of medium from the vessel was prevented by clamping the waste line. This, 'fed-batch,' condition within the vessel appeared to halt lysis and was carried out until the cells had grown to an OD_{600nm} not exceeding 1.2 and was stopped by opening the waste line. The pump was then adjusting to allow fresh medium to flow at the desired rate i.e.. $0.02h^{-1}$, thereby initiating the desired continuous culture conditions.

Other factors were also considered in trying to elucidate the cause of the cell lysis. These were, replacing DL-isoleucine with a new stock, as a batch had been purchased and used the first time that lysis was observed. Also any possibility of electrolytes contained within the pH and dissolved oxygen probes leaking into the culture and causing lysis was discounted by removing both probes from the chemostat as a precautionary measure. These factors were discounted as lysis continued to occur after the changes had been made (not shown).

The problem of cell lysis described above was solved by the 'fed-batch' conditions described, however a second lytic phenomenon was also observed. This problem however appeared to be directly linked to the impeller speed and though the continuous culture had proceeded with the impeller at rheostat setting '2', equivalent to a speed of 360-380 RPM (impeller speed measured using a Venture Microtach 8100 Tachometer), throughout many of the early cultures, a rapid lysis (visible by eye within 2-3 hours) was observed during later runs when the impeller was running at this speed. The lysis could be reversed by reducing the impeller speed to 200 RPM, and though the speeds involved here were unlikely to cause shear effects, in the absence of another reason, cannot be discounted. It is possible that during autoclaving the chemostat vessel that some

of the medium was forced into the impeller bearing housing. Upon repeated sterilisation the medium trapped in the bearing housing forms melanoidins. The presence of these substances in chemostat cultures have been shown to lead to cell lysis (Pfeifer and Vojnovich, 1952), and it is possible that their release into the culture could have been a function of shaft speed. However, the ability of the cells to grow back when the impeller speed was reduced appears to discount melanoidins as the cause of lysis in this instance, and this was supported by the problem persisting after the impeller bearings were replaced and when sterilisation of the spent culture occurred in such a way as to prevent entry of medium into the bearing housing.

Therefore, in subsequent experiments the impeller speed was reduced to 200 RPM, speeds low enough to prevent initiation of the lytic phenomenon but sufficient, with an increase in the supply of air, to maintain levels of oxygenation within the culture (58% (4.42mg O_2 l^{-1}) at 30°C).

Both the instances of cell lysis described here could be further manifestations of the same instability seen with cells in anaerobic culture. As for the anaerobic cultures, in both cases the medium containing the lysed cells were checked for the presence of listerial phage and none were found.

3.3 FATTY ACID AND POLAR LIPID PROFILES OF *L. monocytogenes*

3.3.0 Fatty acid analysis of *L. monocytogenes.*

Investigation of the effect of growth temperature on the fatty acid composition of *L. monocytogenes* NCTC 7973 was performed using gas liquid chromatography (GLC). The cells used in the analysis were cultured in the chemostat before being harvested and stored [See 2.3.5].

Figures 3.6-3.9 show typical GLC traces of the fatty acids (in methyl ester form [See 2.4.3]) of *L. monocytogenes* NCTC 7973 at each of the following growth temperatures 30°C, 20°C, 15°C and 10°C. In addition to the traces shown on Figures 3.6-3.9, percentage values for all FAME samples analysed are given in Appendix I and mean percentage values are given in Table 3.3.

Comparison of the 30°C trace (Figure 3.6) with the retention times of known standards (Figure 3.5), indicated that the major branched chain fatty acids present in *L. monocytogenes* at 30°C are *anteiso-,* in particular *ai*-15:0, and also *ai*-17:0. FAME traces for each of the other growth temperatures also show the presence of *ai*-15:0 as the major fatty acid (Figures 3.7-3.9). In addition however, the same traces also show a visible decrease in the peak size (and therefore quantity) of *ai*-17:0 with a decrease in growth temperature (Figures 3.9-3.7). Mean percentage fatty acid values given in Table 3.3 confirms *ai*-15:0 as the major fatty acid present at all temperatures, and also shows a decrease in levels of *ai*-17:0 from 18.1% of total fatty acids at 30°C, to 5.3% at 10°C. Table 3.3 also shows that the amount of *i*-15:0 present decreased with a reduction in growth temperature (from 3.3% of total fatty acids at 30°C, to 1.4% at 10°C). A reduction in growth temperature did not however, appear to effect amounts of *ai*-15:0, or *i*-16:0 present.

In addition to the effect of different growth temperatures on the fatty acids listed above, there were two other sets of fatty acid data presented in Table 3.3. These refer to FAME peaks that were either present in small quantities and/or peaks that could not be accurately identified using the FAME standards. All fatty acids falling in these categories were grouped as being ≤15 carbons or ≥16 carbons in length. A reduction in growth temperature increased the quantity of fatty acids ≤15 carbons in length (From 7.3% of total fatty acids at 30°C, to 21.8% at 10°C) whilst the fatty acids grouped ≥16 carbons remained constant at all growth temperatures (Table 3.3).

It is clear from Table 3.3 that an increase in the small chain fatty acids (i.e. those grouped as ≤15 carbons in length) of *L. monocytogenes* NCTC 7973 is an important adaptation to growth at low temperature. In view of this a more detailed comparison of the fatty acids ≤15 carbons in length was made and the results are indicated in Table 3.4.

Table 3.4 shows differences in the fatty acids grouped as ≤15 carbons in length for two samples, HP13 (30°C) and HP15 (10°C). Only two growth temperatures were compared because the largest difference in fatty acid composition was seen between these temperatures (Table 3.3). Also, the relative amounts of fatty acids present in the ≤15carbon grouping remain fairly constant as the growth temperature was reduced from 30°C to 15°C. A marked increase in the amount of fatty acids ≤15 carbons in length was seen only at the lowest growth temperature studied (10°C) (Table 3.3). Samples HP13 and HP15 were compared in Table 3.4 as they had the greatest numbers of fatty acids ≤15 carbons in length. Although only two samples are compared in Table 3.4, the individual fatty acids ≤15 carbons in length present in every FAME sample prepared from cells grown at 30°C and 10°C are listed in Appendix II.

The fatty acids labelled 'a' to 'j' in Table 3.4 refer to the FAME peaks for HP13 and HP15, and these peaks are also shown on Figures 3.6 and 3.9. It is important to note that although many of these were common to all the samples analysed, additional peaks were also seen in other samples, particularly those from cells grown at 10°C [See Appendix II]. Retention times are shown in Table 3.4 to ensure that the same FAME peaks are compared at both growth temperatures and also for identification (where possible) by comparison with the FAME standards shown in Figure 3.5.

Although the FAME standards employed in the study did not permit the identification of the majority of fatty acids shown in Table 3.4 and Appendix II, it is clear from Table 3.4 that, with the exception of two fatty acids, the quantity of the individual fatty acids increased significantly at a growth temperature of 10°C. Growth at 10°C increased the amount of 12:0 (Peak 'c') produced (2.2% of total fatty acids at 30°C, to 5.1% at 10°C(Table 3.4)) and also 14:0 (Peak 'i') was only detected in cells grown at 10°C (Table 3.4, Appendix II). In addition, quantities of five other fatty acids with retention times not exactly matching those of known standards (designated peaks 'a', 'd'-'h' in Table 3.4) all showed an increase in response to a reduction in growth temperature. A reduction in growth temperature stopped production of 2OH-14:0 (Peak 'j') by *L. monocytogenes,* and also reduced the amount of a fatty acid designated 'b' in Table 3.4: 2.7% of total fatty acid at 30°C to 1.3% at 10°C. Although the accurate identification of all the fatty acids present in the ≤15 carbons group was not possible, the nearest missing standard to each, in terms of carbon number, is given in Table 3.4.

Appendix II lists fatty acids ≤15 carbons in length for all FAME samples prepared from cells grown at 10°C and 30°C. It is clear from Appendix II that comparison of individual samples indicates some variation in the fatty acids ≤15 carbons in length present. This is not easily explained, but may be due to slight variations in the esterfying procedure. Of the fatty acids present in the samples

compared in Table 3.4, the majority however, occur in other samples [See Appendix II]. The two exceptions are fatty acid 'e' (Table 3.4), which occurs in all samples prepared from cells grown at 10°C, but only in a single preparation from cells grown at 30°C (HP13), and fatty acid 'b' (Table 3.4) which occurs in HP13 (30°C) and HP15 (10°C) only [See Appendix II].

Although discrepancies do exist between the individual samples, the data presented in Tables 3.3-4 and Appendix I and II clearly indicate that an increase in the amount of lower chain fatty acids synthesised by *L. monocytogenes* NCTC 7973 is an important response to cold stress.

Key to Figure 3.5

			Retention time (min)
1.	11:O	Methyl undecanoate	16.8
2.	2-OH 10:0	Methyl 2-hydroxydecanoate	16.98
3.	12:0	Methyl dodecanoate	19.45
4.	13:0	Methyl tridecanoate	22.68
5.	2-OH 12:0	Methyl 2-hydroxydodecanoate	22.93
6.	3-OH 12:0	Methyl 3-hydroxydodecanoate	23.64
7.	14:0	Methyl tetradecanoate	25.41
8.	*i*-15:0	Methyl 13-methyltetradecanoate	27.09
9.	*ai*-15:0	Methyl 12-methyltetradecanoate	27.29
10.	15:0	Methyl pentadecanoate	28.01
11.	2-OH 14:0	Methyl 2-hydroxytetradecanoate	28.33
12.	3-OH 14:0	Methyl 3-hydroxytetradecanoate	28.98
13.	*i*-16:0	Methyl 14-methylpentadecanoate	29.63
14.	$16:1^{9}$	Methyl cis-9-hexadecenoate	29.91
15.	16:0	Methyl hexadecanoate	30.51
16.	*i*-17:0	Methyl 15-methylhexadecanoate	32.05
17.	17:0Δ	Methyl cis-9, 10-methylenehexadecanoate	32.49
18.	17:0	Methyl heptadecanoate	32.89
19.	2-OH 16:0	Methyl 2-hydroxyhexadecanoate	33.26
20.	$18:2^{9,12}$	Methyl cis-9, 12,-octadecadienoate	34.31
21.	$18:1^{9}$	Methyl trans-9-octadecenoate	34.52
22.	$18:1^{9}$	Methyl trans-9-octadecenoate &	34.66
	$18:1^{11}$	Methyl cis-11-octadecenoate	
23.	18:0	Methyl octadecanaoate	35.17
24.	19:0Δ	Methyl cis-9,10,-methyleneoctadecanoate	37.02
25.	19:0	Methyl nonadecanoate	37.37
26	20:0	Methyl eicosanoate	39.46

Figure 3.5 Typical GLC trace showing bacterial FAMES standard mix.

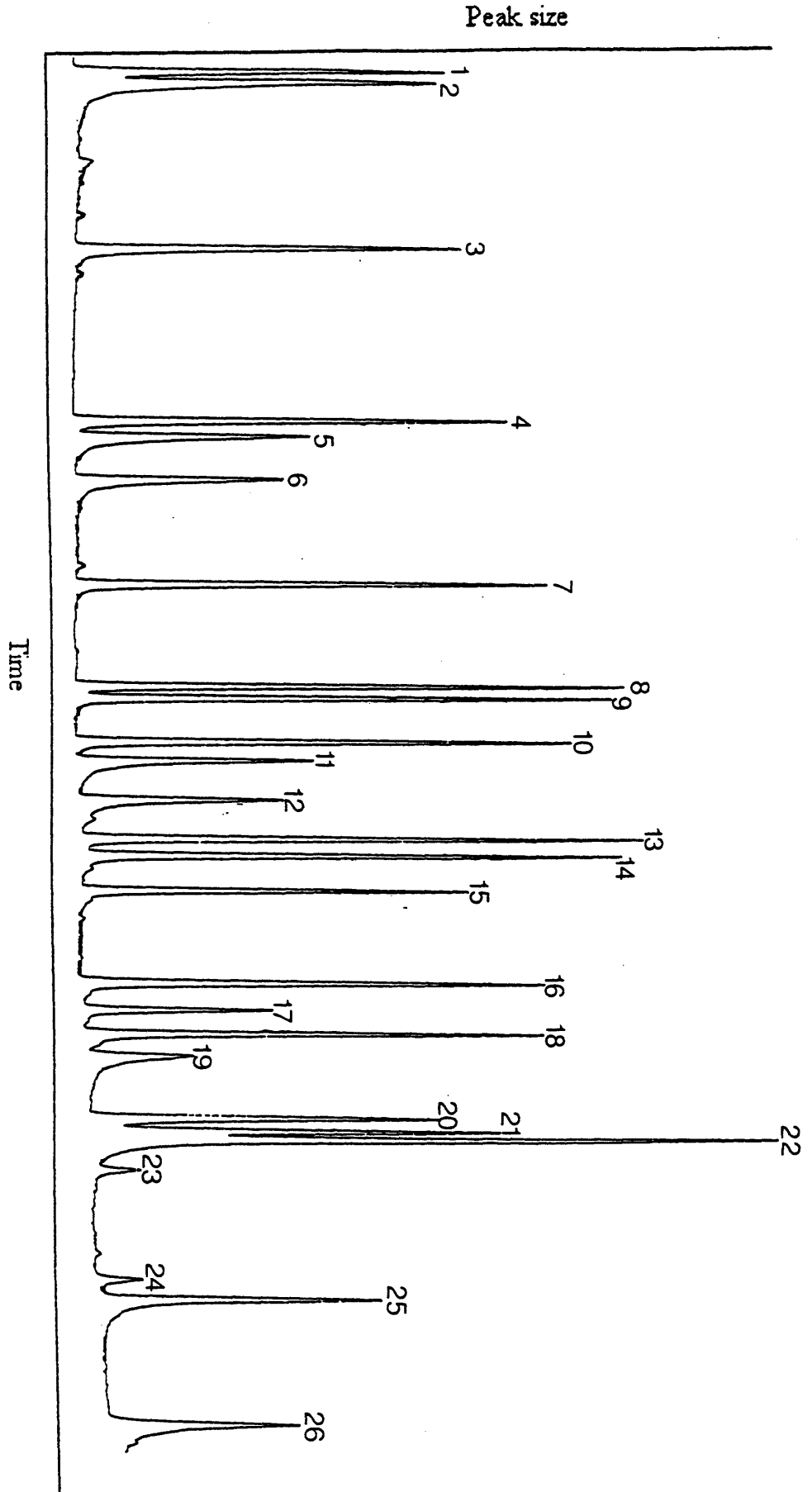


Figure 3.6 Example of GLC trace showing FAMES of *L. monocytogenes* at 30°C. Trace shown is for Sample HP13, one of six samples the data for which are listed in Appendix I. Peaks 'a'-'j' refer to FAME peaks ≤15 carbons in length, [See Table 3.4, Appendix II].

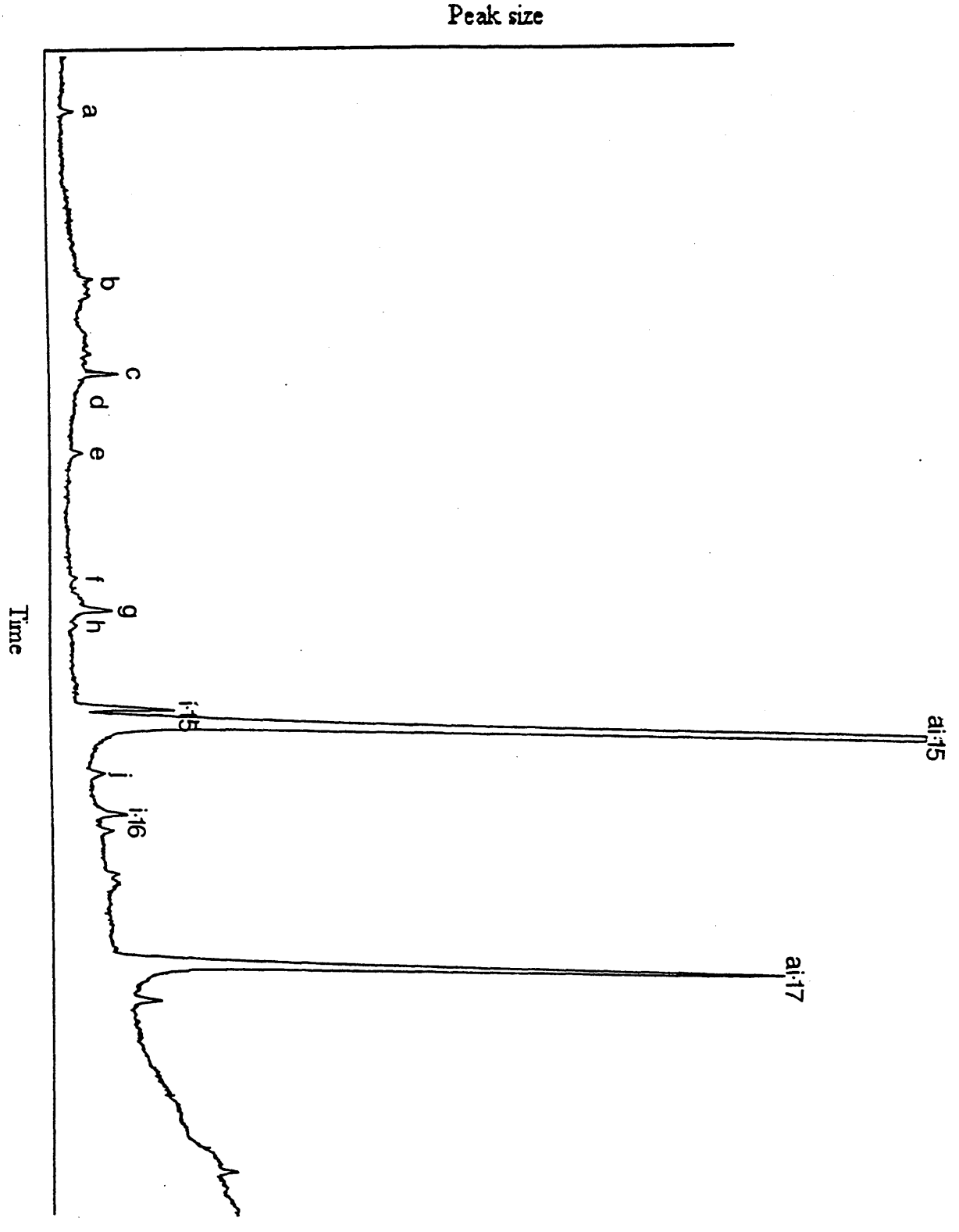


Figure 3.7 Example of GLC trace showing FAMES of *L. monocytogenes* at 20°C. Trace shown is for Sample HP17 one of five samples the data for which are listed in Appendix I.

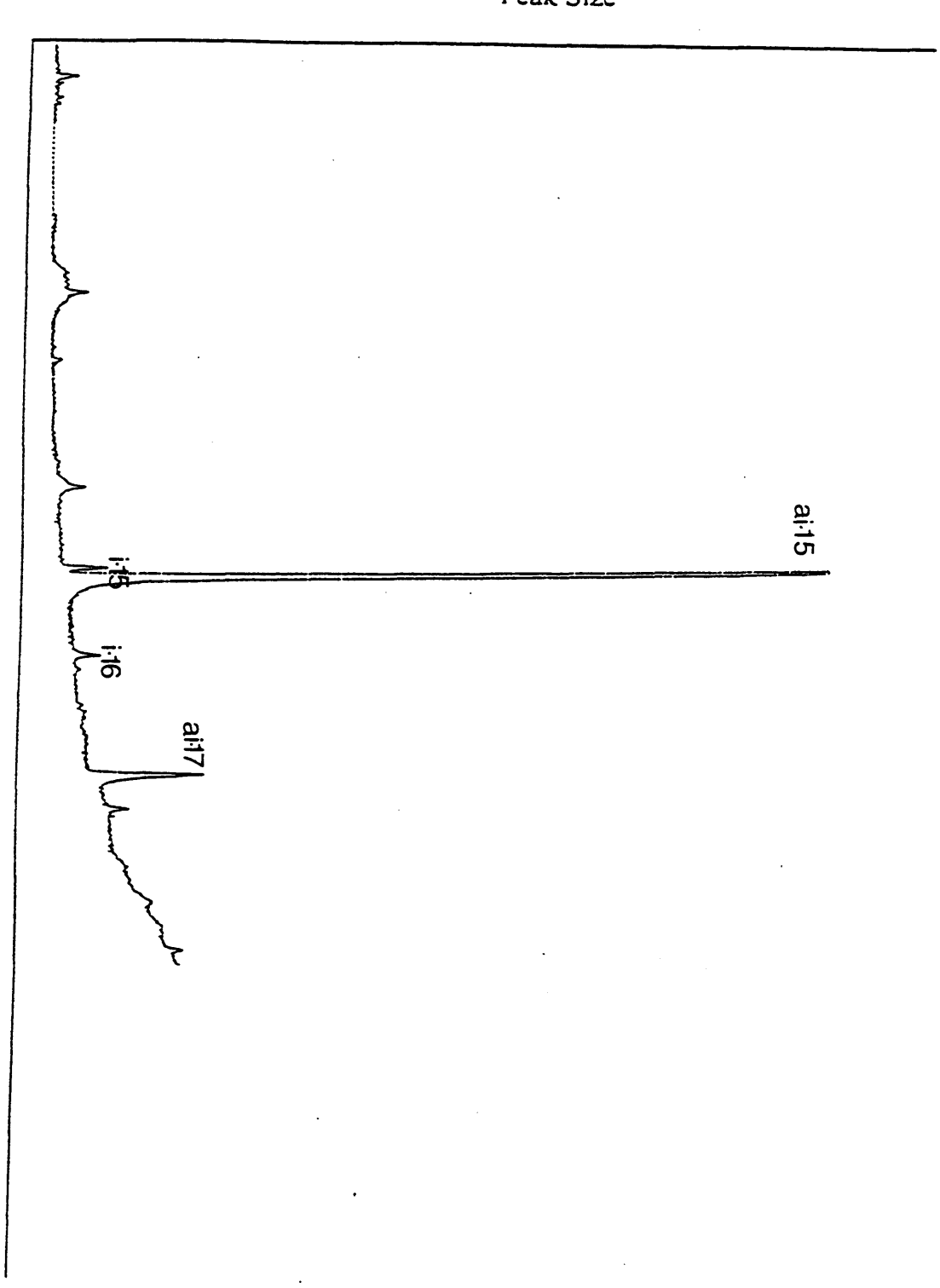


Figure 3.8 Example of GLC trace showing FAMES of *L. monocytogenes* at 15°C. Trace shown is for Sample HP16 one of four samples the data for which are listed in Appendix I.

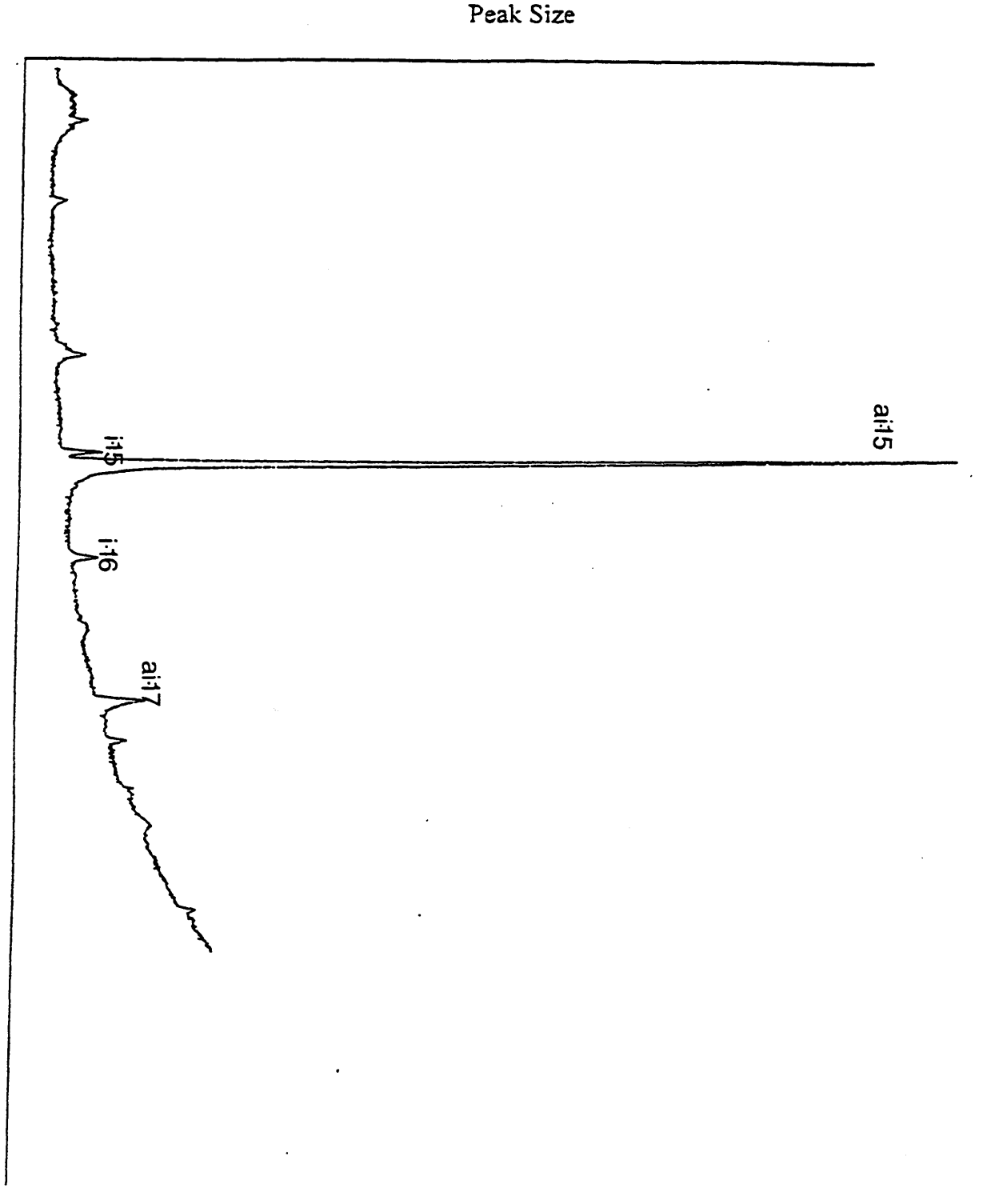


Figure 3.9 Example of GLC trace showing FAMES of *L. monocytogenes* at 10°C. Trace shown is for Sample HP15, one of six samples the data for which are listed in Appendix I. Peaks 'a'-'j' refer to FAME peaks ≤15 carbons in length, [See Table 3.4, Appendix II].

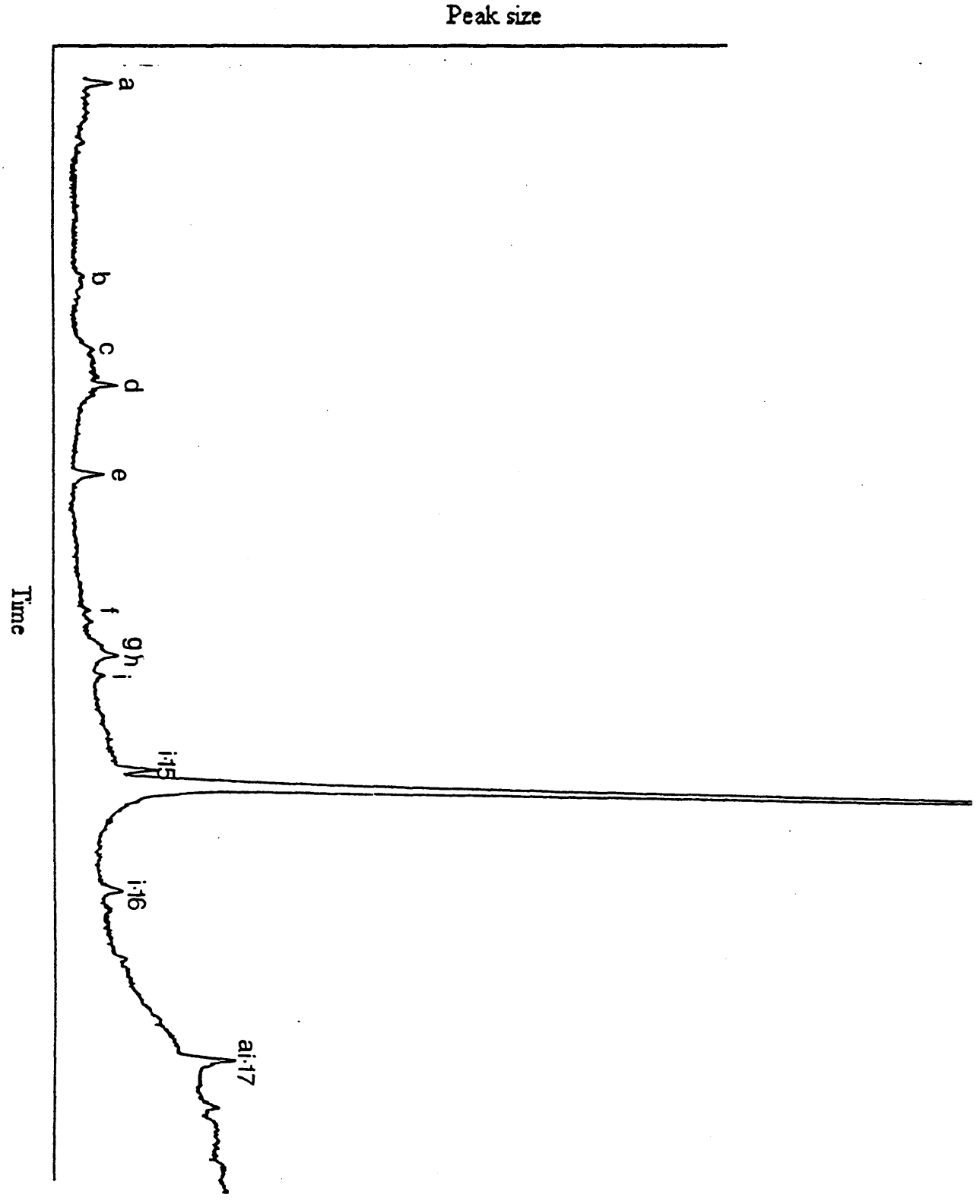


Table 3.3 Predominant fatty acids in *L. monocytogenes* NCTC 7973, as a percentage of total fatty acids, at a range of growth temperatures.

Temp.\ FAMES	≤15	i-15:0	ai-15-0	i-16:0	ai-17:0	≥16
30°C	7.3	3.3	64.1	2.4	18.1	4.2
20°C	12.8	3.0	71.2	0.9	10.5	1.6
15°C	9.7	1.6	71.6	2.2	6.3	4.9
10°C	21.8	1.4	64.3	2.4	5.3	4.9

Values are the mean of FAME preparations from three separate sets of continuously cultured cells at each temperature (The raw data for every FAME preparation at each temperature are listed in Appendix I).

Table 3.4 Retention times of individual fatty acids grouped less than or equal to 15 carbons in length in samples HP13 and HP15, from cells grown at 30°C and 10°C respectively.

FAME Peak[1]	Sample HP13 (30°C)		Sample HP15 (10°C)		Fatty Acid Type
	%	**Rt**	**%**	**Rt**	
a	0.2	14.85	1.6	14.82	<11C ↑[2]
b	2.7	18.35	1.3	18.3	NK[a] ↓
c	**2.1**	**19.43**	**5.1**	**19.5**	**12:0 ↑**
d	1.7	20.23	3.4	20.2	NK[a] ↑
e	0.2	21.9	1.5	21.87	NK[b] ↑
f	0.1	24.47	1.4	24.29	NK[c] ↑
g	0.6	24.94	1.5	25.0	NK[c] ↑
h	0.9	25.11	1.9	25.06	NK[c] ↑
i	**0**	**ND**	**0.3**	**25.41**	**14:0 ↑**
j	**0.33**	**28.47**	**0**	**ND**	**2OH-14:0↓**

[1]Letters 'a'- 'j' refer to FAME peaks ≤15 carbons in length in samples HP13 and HP15 [See Figure 3.6 and 3.9].
[2]Arrows indicate an increase or decrease in the amount of individual fatty acids in response to a reduction in growth temperature.
Bold characters indicate where a firm identification of fatty acids by means of Rt comparison with FAME standards was possible (See Figure 3.5).
[a]Retention time near to that of 12C fatty acids.
[b]Retention time near to that of 13C fatty acids.
[c]Retention time near to that of 14C fatty acids.
%: percentage of total fatty acids.
Rt: Retention time (min).
ND: not determined.
NK: not known.

3.3.1 Analysis of polar lipid profiles of *L. monocytogenes*

A qualitative analysis of polar lipids of *L. monocytogenes* was performed using two-dimensional thin layer chromatography (TLC). Each TLC experiment was repeated at least twice using a different set of continuously cultured cells.

Figures 3.10(a)-(c) shows the polar lipids of *L. monocytogenes,* at three different growth temperatures (30°C, 20°C, and 10°C), stained using dodecamolybdophosphoric acid, a non-specific stain that visualises all polar lipids (Ross *et al.,* 1981). The pattern of spots was the same at each of the three temperatures. Two polar lipids were provisionally identified by comparison with standards, as diphosphatidylglycerol (DPG) and phospha-tidylglycerol (PG). This is can be seen by comparison of polar lipid spots present on Figures 3.10(a)-(c), with the mixture of DPG and PG standards shown on Figure 3.11(d). The identification of the two other polar lipids (spots '1' and '2') seen on Figures 3.10(a)-(c) was attempted using stains specific for individual polar moieties.

The polar lipid spots seen on Figure 3.11 (a)-(c) were visualised using a stain specific for phosphate groups (Zinzadze's Reagent (Dittmer and Lester, 1964), with which a blue coloured spot indicates a positive result). These plates indicate the presence of three phosphate-containing polar lipids, the polar lipids identified as DPG and PG by comparison with standards (Figure 3.11(d)) and an additional unidentified phosphate containing polar lipid designated '3'. All three spots were present at each temperature tested.

Figure 3.12(a)-(c) shows the presence of polar lipids containing an amino group, using ninhydrin, a specific stain for amino groups (Consden and Gordon, 1948). These plates indicate the presence of a single amino-containing polar lipid, designated '4', at each of the study temperatures. Figure 3.12(b) also shows a second amino- containing polar lipid, designated '5'.The other spots present on the Figures 3.12(a) and (b) also deserve comment. Spot 'a' is likely to be a polar

lipid that does not have an amino group present as it is brown in colour, rather than the purple-violet colour typical of an amino group seen when ninhydrin is used. Also the spot designated 'b' on Figure 3.12(a)-(c) may be a free amino acid that has not been removed by the extraction procedure. This conclusion is reached as it appears not to have run in the second phase and polar lipids would be expected to run in the both phases.

Figure 3.13(a)-(c) shows plates sprayed with α-naphthol, a differentiating stain used to identify any sugar moieties present (Jacin and Mishkin, 1965). All the plates indicate the presence of two glycolipids (designated ('6' and '7')) present at each growth temperature. (Figures 3.13 (a)-(c)). A further glycolipid (spot '8') was seen at 10°C only (Figure 3.13(c)). This glycolipid (spot '8') seen at 10°C was not seen at either of the other temperatures examined (Figure 3.13(a), (b)) even when large quantities of the sample were 'spotted' onto the plates. However, it was not seen at 10°C when the non-specific stain (dodecamolybdophosphoric acid) was used (See Figure 3.10(c)). Two additional spots were also seen. Spot 'c' which ran poorly in both phases, may be a free sugar contaminant that was not removed during the extraction. Spot 'd' was seen in extracts of cells cultured at 30°C and 10°C (Figures 3.13(a) and (c)). This appears to be a non-sugar containing polar lipid, because the spot was brown rather than the purple-blue colour characteristic of a glycolipid using α-naphthol (Jacin and Mishkin, 1965).

The use of individual stains (Figures 3.11-3.13) shows additional spots to those seen using the non-specific stain (Figure 3.10). Although some of these spots are present when more than one specific stain is used, some are unique to a particular stain. Nevertheless, an attempt was made to identify all the spots from the data given above, and this is discussed below:

The aminolipid, designated spot '5' (Figure 3.12(b) was not a repeatable finding and may be an artefact of the polar lipid extraction for the sample shown in Figure 3.12(b).

Spot 'd' (Figure 3.13 (a) and (c)) is interpreted to be PG (Figure 3.10, 3.11), as their positions are similar. The non-appearance of spot 'd' on Figure 3.13 (b) is not easy to explain, although it may be due to differences in times the plates were exposed to 120°C. Occasionally a slightly longer period than the 10 minutes at 120°C stated in Section 2.4.6 was employed to improve visibility. Another experiment using cells grown in the chemostat at 20°C (not shown) indicated a very small (trace) amount of a lipid spot equivalent to spot 'd' when sprayed with α-naphthol.

The phospholipid designated '3' (in Figure 3.11(a)-(c)) appears to be the same as that designated spot '2' on Figure 3.10 because their positions relative to PG and DPG are similar. This phospholipid also appears to be present as spot 'a' on Figure 3.12(a) and (b).

Glycolipid spot '6' on Figure 3.13 appears to be the same spot as that numbered '1' on Figure 3.10. The position of spot 'd', a non-sugar containing polar lipid, which is probably PG, (see above) on Figure 3.13 (a) and (c) enables this tentative identification.

The above statements are based on a comparison of the positions of the two unknown polar lipids identified using the 'catch all' stain (Figure 3.10) with those of polar lipids identified using specific stains (Figures 3.11-3.13). These, together with the relative positions of spots 'a' and 'd', indicate that the two uncharacterised polar lipids present on Figure 3.10 are a phospholipid (spot '2') and a glycolipid (spot '1'). In addition, the appearance of the phospholipid (spot '2') as a non-amino- containing polar lipid on Figures 3.12 (a),(b) may aid the

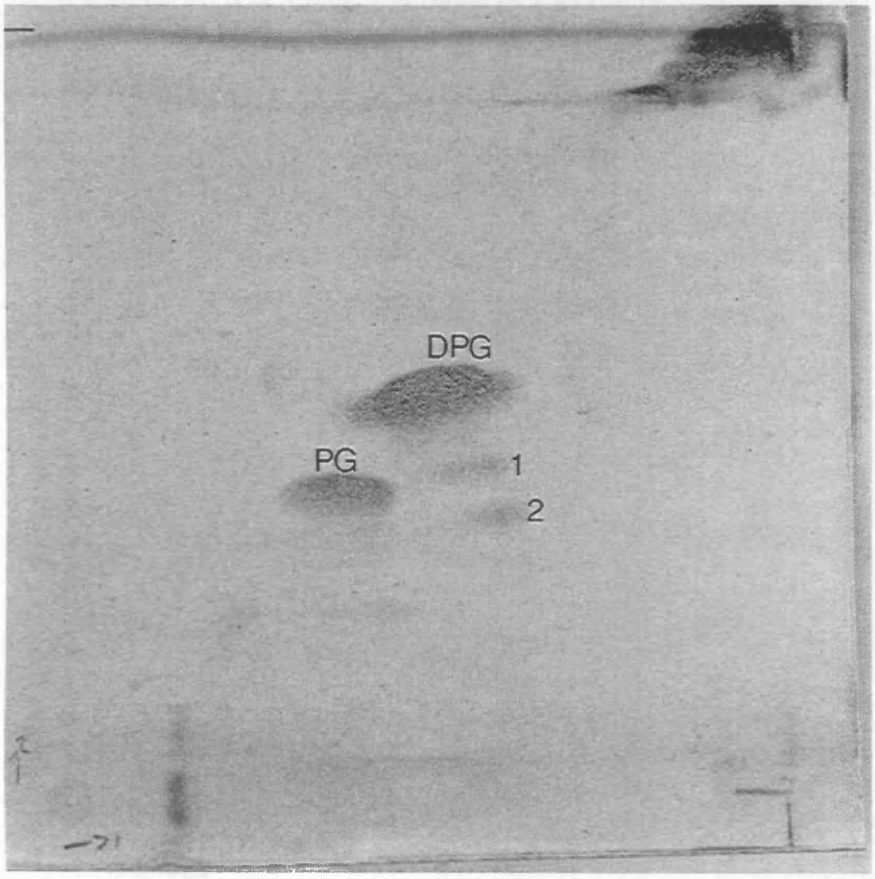
identification of the amino- containing polar lipid, spot '4' seen on Figures 3.12(a)-(c). If glycolipid (spot '1') on Figure 3.10(a)-(c), is also the unknown polar lipid, spot 'a' on Figure 3.12(a),(b), then it follows that the position of spot '4' indicates that it is the polar lipid designated as spot '1' (Figure 3.10(a)-(c)), thereby showing the possibility of an amino-glycolipid.

This analysis of the spots still leaves two lipid spots (spots 7 and 8) identified on the basis of the specific stains, but not seen by the 'catch all'. Differences in the sensitivity of the stains could account for this observation.

The tentative identification of the polar lipids of *L. monocytogenes* from this study are listed in Table 3.5. This table indicates that PG, DPG a phospholipid and two glycolipids, one possibly containing an amino- group, are present at all growth temperatures. The occurrence of only one polar lipid appears to be temperature dependant, this is a third glycolipid (spot '8') seen at 10°C only.

Figure 3.10 Polar lipids of *L. monocytogenes* NCTC 7973. The photograph shows a plate that has been sprayed with a non-specific polar lipid stain (Dodecamolybdophosphoric acid). Identification of the individual lipids was made using either specific stains or by comparison with standards [See Section 3.1].

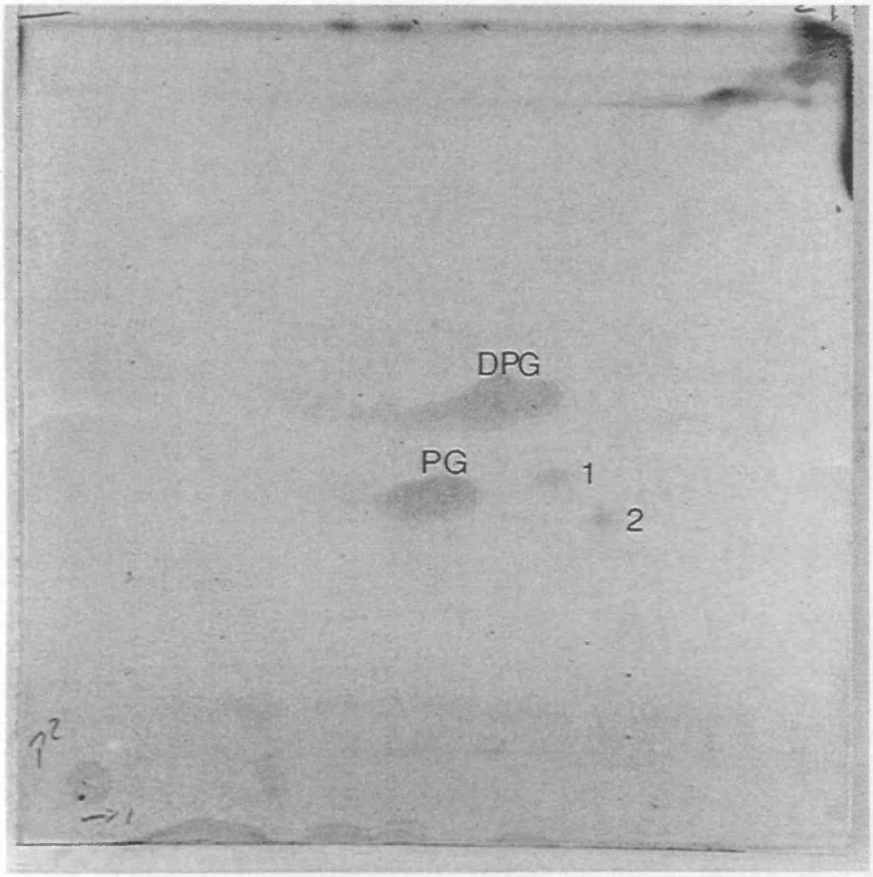
Figure 3.10 (a) Cells grown at 30°C.



Key
DPG. Diphosphatidylglycerol.
PG. Phosphatidylglycerol.
1. Polar lipid.
2. Polar lipid.
Arrows on the bottom left-hand corner of the plate indicates the direction of the 1st and 2nd phase.

Figure 3.10 Polar lipids of *L. monocytogenes* NCTC 7973. The photograph shows a plate that has been sprayed with a non-specific polar lipid stain (Dodecamolybdophosphoric acid). Identification of the individual lipids was made using either specific stains or by comparison with standards [See Section 3.1].

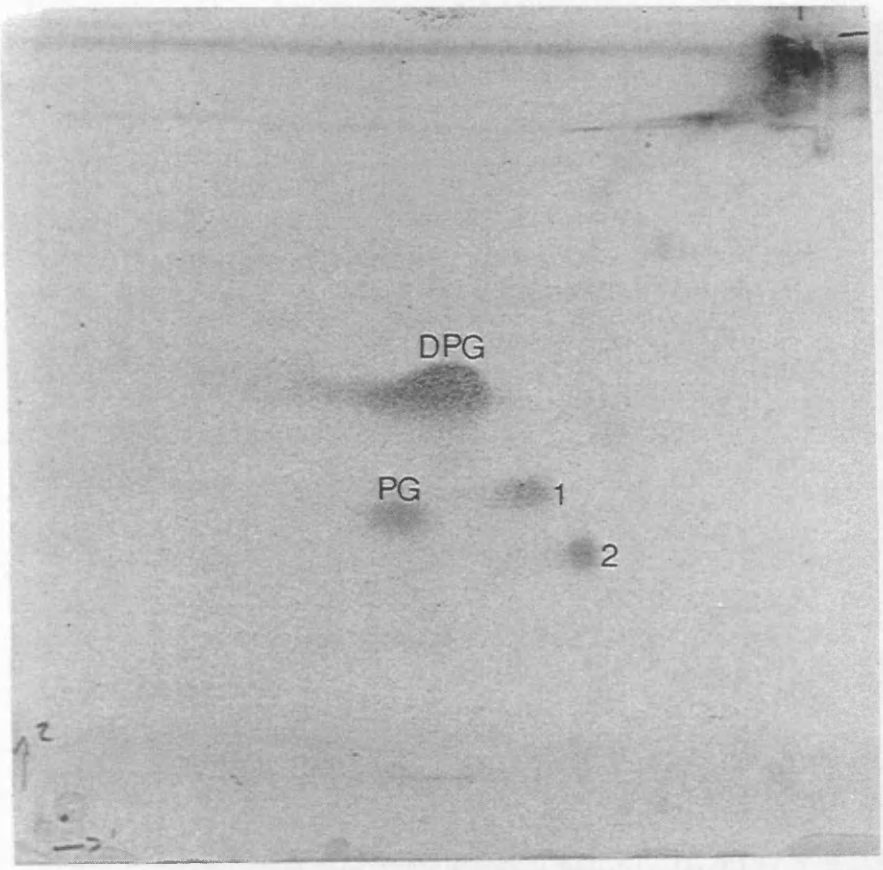
Figure 3.10 (b) Cells grown at 20°C.



Key
DPG. Diphosphatidylglycerol.
PG. Phosphatidylglycerol.
1. Polar lipid.
2. Polar lipid.
Arrows on the bottom left-hand corner of the plate indicates the direction of the 1st and 2nd phase.

Figure 3.10 Polar lipids of *L. monocytogenes* NCTC 7973. The photograph shows a plate that has been sprayed with a non-specific polar lipid stain (Dodecamolybdophosphoric acid). Identification of the individual lipids was made using either specific stains or by comparison with standards [See Section 3.1].

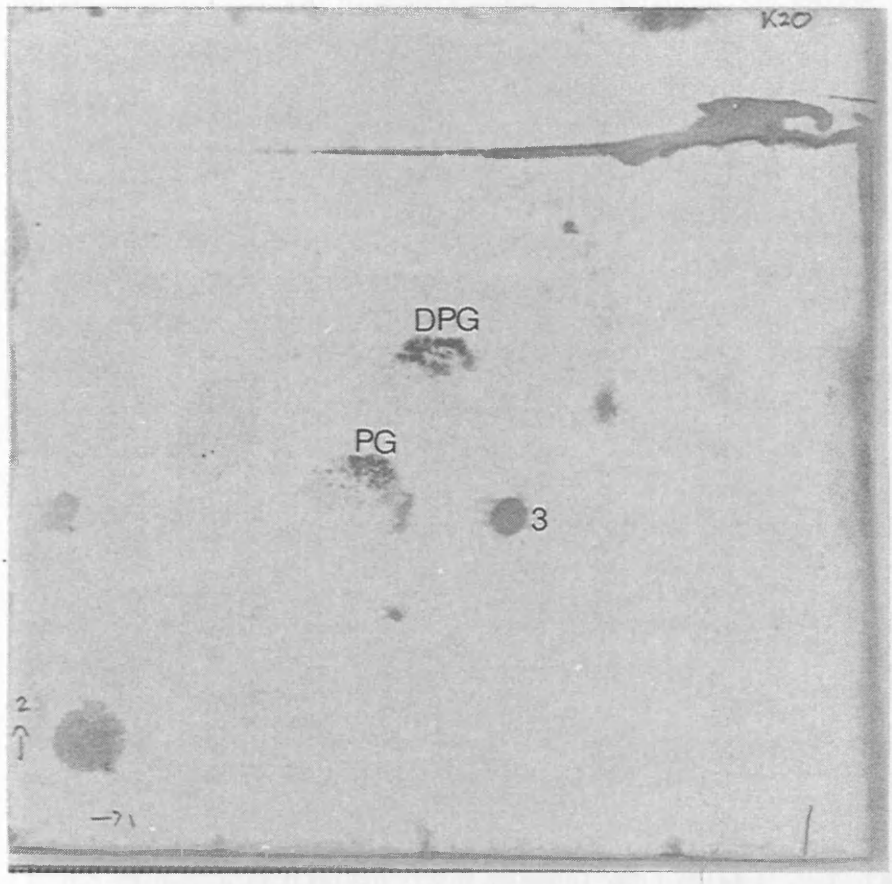
Figure 3.10 (c) Cells grown at 10°C.



Key
DPG. Diphosphatidylglycerol.
PG. Phosphatidylglycerol.
1. Polar lipid.
2. Polar lipid.
Arrows on the bottom left-hand corner of the plate indicates the direction of the 1st and 2nd phase.

Figure 3.11 Identification of phosphate groups present on polar lipids at each of the three growth temperatures studied. The presence of phosphate groups were identified by spraying with a specific stain (Zinzadze's Reagent). Due to the presence of concentrated acid in the reagent all plates shown are in poor condition and as a result only spots coloured blue, i.e. positive for the presence of phosphate groups are shown on the keys.

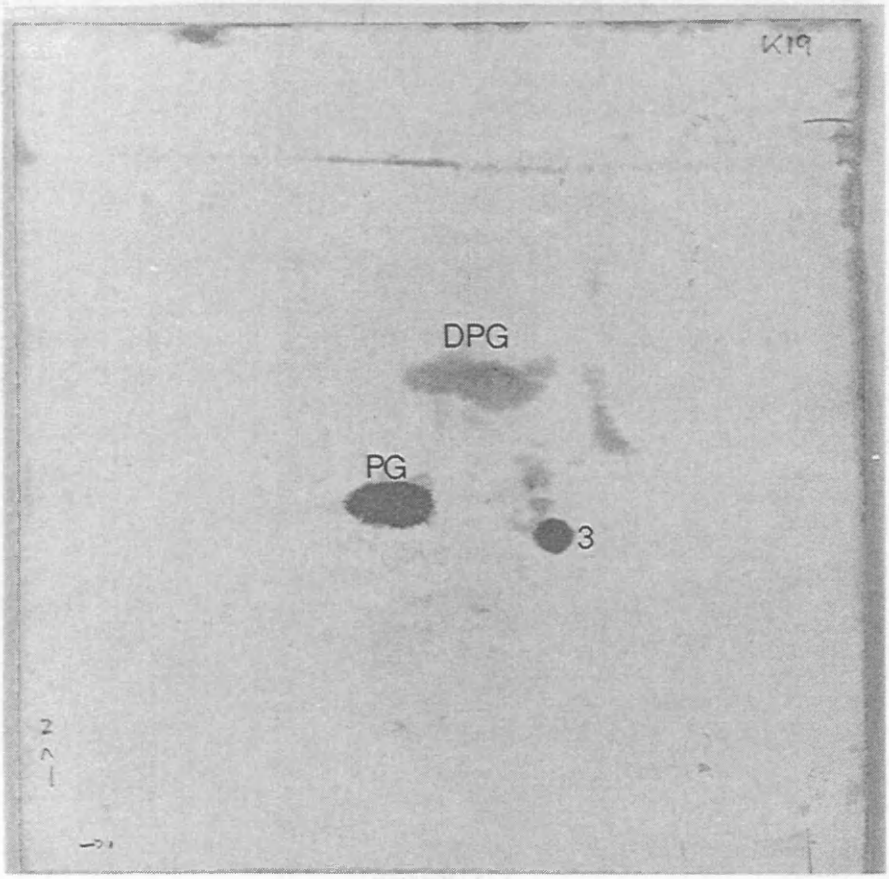
Figure 3.11 (a) Cells grown at 30°C.



Key
DPG. Diphosphatidylglycerol (blue).
PG. Phosphatidylglycerol (blue).
3. Phospholipid (blue).
Arrows on the bottom left-hand corner of the plate indicates the direction of the 1st and 2nd phase.

Figure 3.11 Identification of phosphate groups present on polar lipids at each of the three growth temperatures studied. The presence of phosphate groups were identified by spraying with a specific stain (Zinzadze's Reagent). Due to the presence of concentrated acid in the reagent all plates shown are in poor condition and as a result only spots coloured blue, i.e. positive for the presence of phosphate groups are shown on the keys.

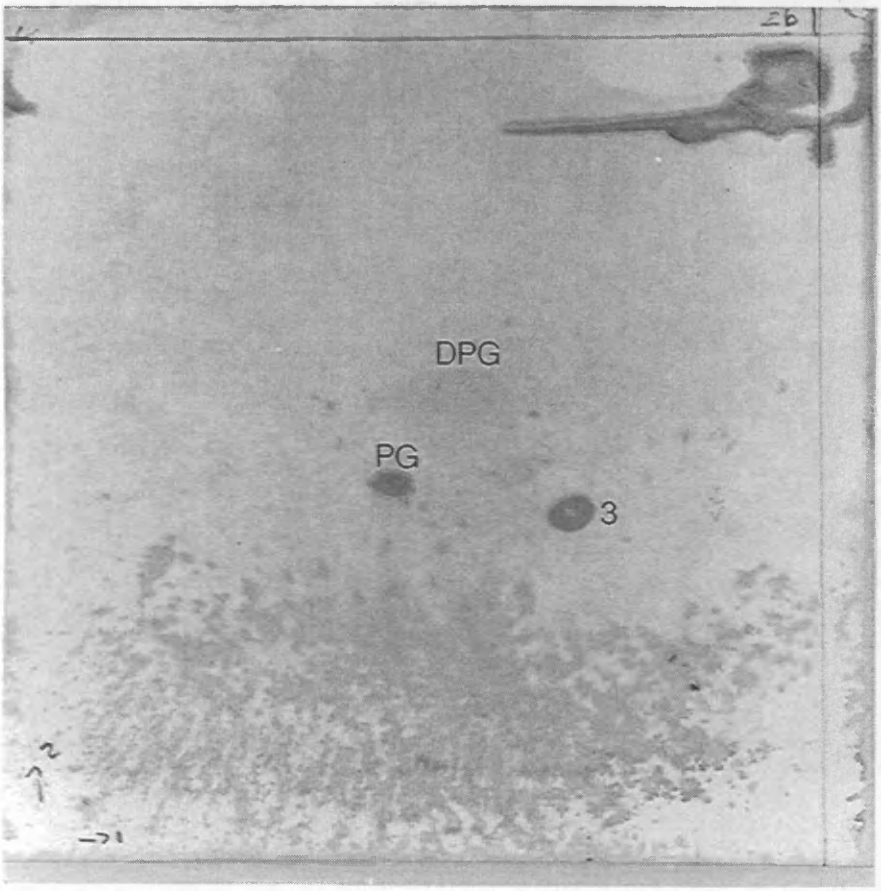
Figure 3.11 (b) Cells grown at 20°C.



Key
DPG. Diphosphatidylglycerol (blue).
PG. Phosphatidylglycerol (blue).
3. Phospholipid (blue).
Arrows on the bottom left-hand corner of the plate indicates the direction of the 1st and 2nd phase.

Figure 3.11 Identification of phosphate groups present on polar lipids at each of the three growth temperatures studied. The presence of phosphate groups were identified by spraying with a specific stain (Zinzadze's Reagent). Due to the presence of concentrated acid in the reagent all plates shown are in poor condition and as a result only spots coloured blue, i.e. positive for the presence of phosphate groups are shown on the keys.

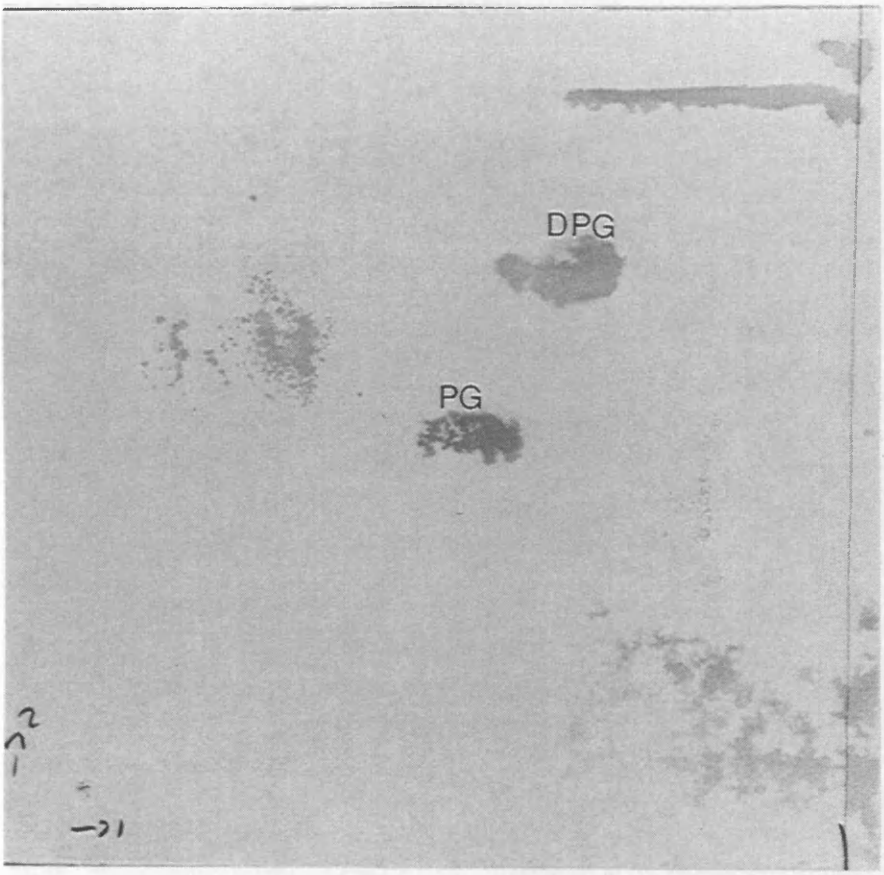
Figure 3.11 (c) Cells grown at 10°C.



Key

DPG. Diphosphatidylglycerol (blue).
PG. Phosphatidylglycerol (blue).
3. Phospholipid (blue).
Arrows on the bottom left-hand corner of the plate indicates the direction of the 1st and 2nd phase.

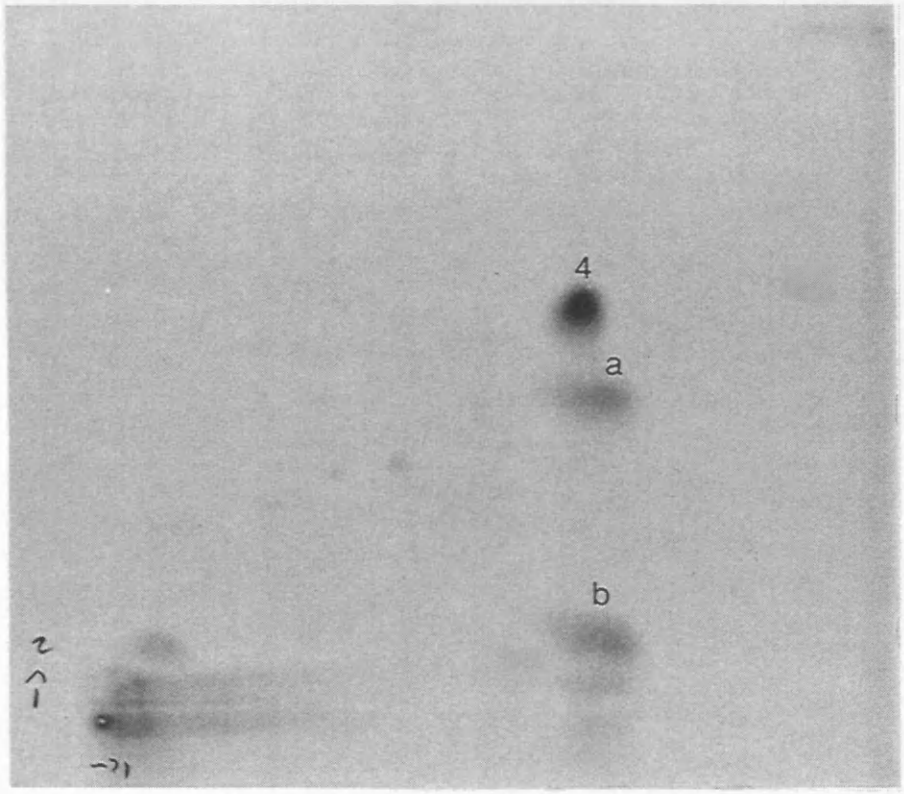
Figure 3.11 (d) Diphosphatidylglycerol and phosphatidylglycerol standards visualised using Zinzadze's reagent. The relative position of the lipids on this plate provisionally indicates the presence of these polar lipids in this study, as has been noted previously for *L. monocytogenes* [See Section 3.1].



Key
DPG. Diphosphatidylglycerol (blue).
PG. Phosphatidylglycerol (blue).
Arrows on the bottom left-hand corner of the plate indicates the direction of the 1st and 2nd phase.

Figure 3. 12 Identification of amino groups present on polar lipids (Specific stain used: ninhydrin). Spots staining purple-violet were identified as aminolipids, and other polar lipids present on the plates do not contain amino- moieties, as they are the incorrect colour (brown).

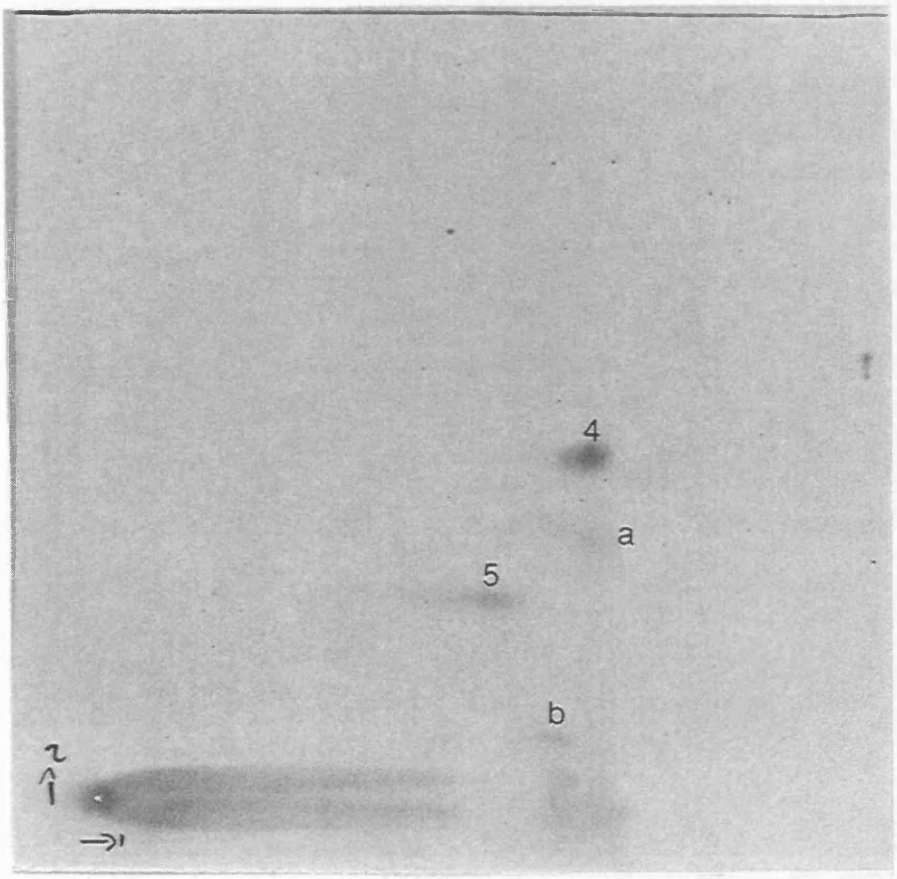
Figure 3.12 (a) Cells grown at 30°C.



Key
4. Aminolipid (purple-violet).
a. Polar lipid with no amino group because spot is brown, rather than purple-violet [See Section 3.1].
b. Free amino-acid, [See Section 3.1].
Arrows on the bottom left-hand corner of the plate indicates the direction of the 1st and 2nd phase.

Figure 3. 12 Identification of amino groups present on polar lipids (Specific stain used: ninhydrin). Spots staining purple-violet were identified as aminolipids, and other polar lipids present on the plates do not contain amino- moieties as they are the incorrect colour (brown).

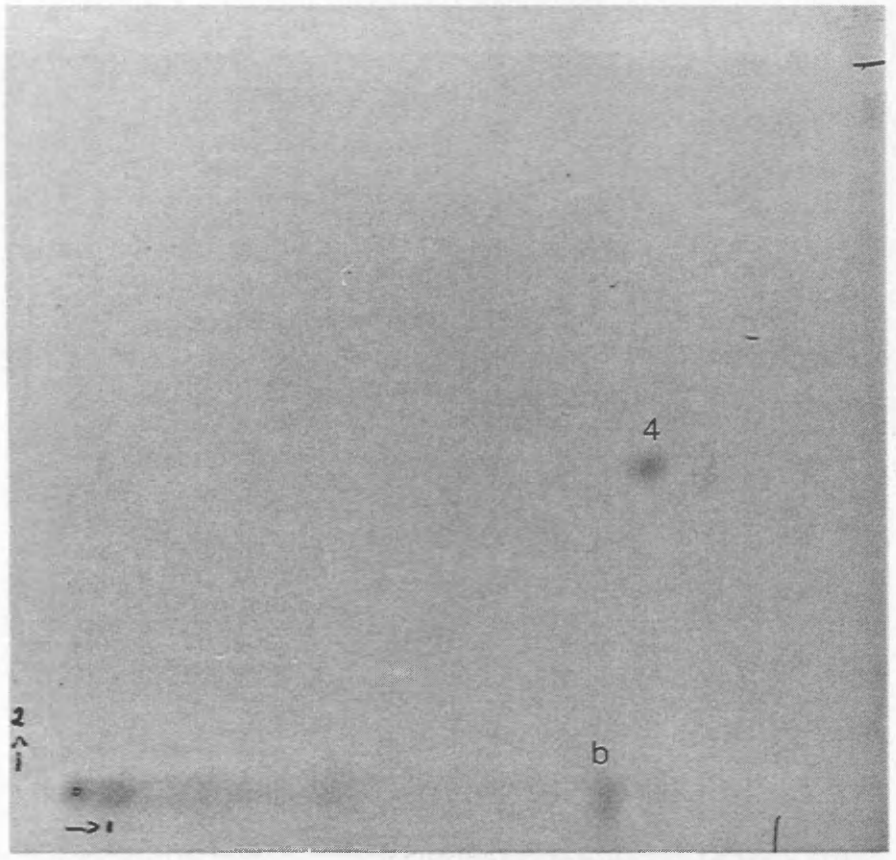
Figure 3.12 (b) Cells grown at 20°C.



Key
4. Aminolipid (purple-violet).
5. Aminolipid (purple-violet).
a. Polar lipid with no amino group because spot is brown, rather than purple-violet [See Section 3.1].
b. Free amino-acid, [See Section 3.1].
Arrows on the bottom left-hand corner of the plate indicates the direction of the 1st and 2nd phase.

Figure 3. 12 Identification of amino groups present on polar lipids (Specific stain used: ninhydrin). Spots staining purple-violet were identified as aminolipids.

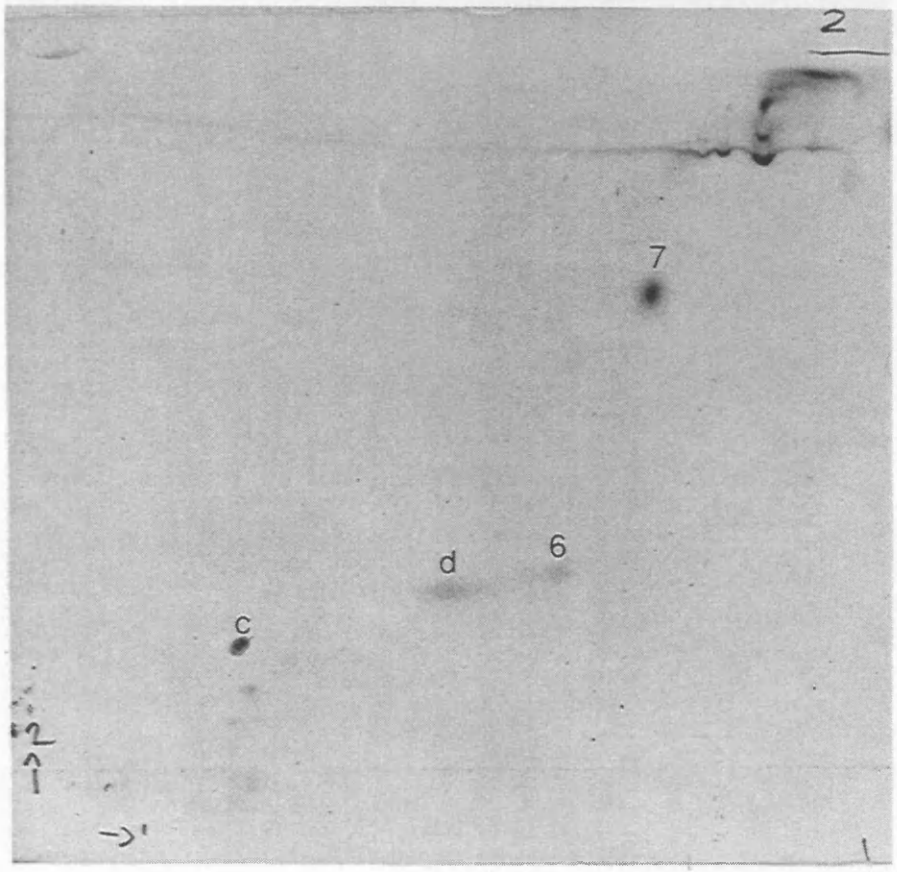
Figure 3.12 (c) Cells grown at 10°C.



Key
4. Aminolipid (purple-violet).
b. Free amino-acid, [See Section 3.1].
Arrows on the bottom left-hand corner of the plate indicates the direction of the 1st and 2nd phase.

Figure 3.13 Identification of a polar lipids containing sugar moieties (using α-naphthol stain). Spots staining purple-blue were identified as glycolipids and other polar lipids present on the plates, do not contain sugar moieties as they are the incorrect colour (brown).

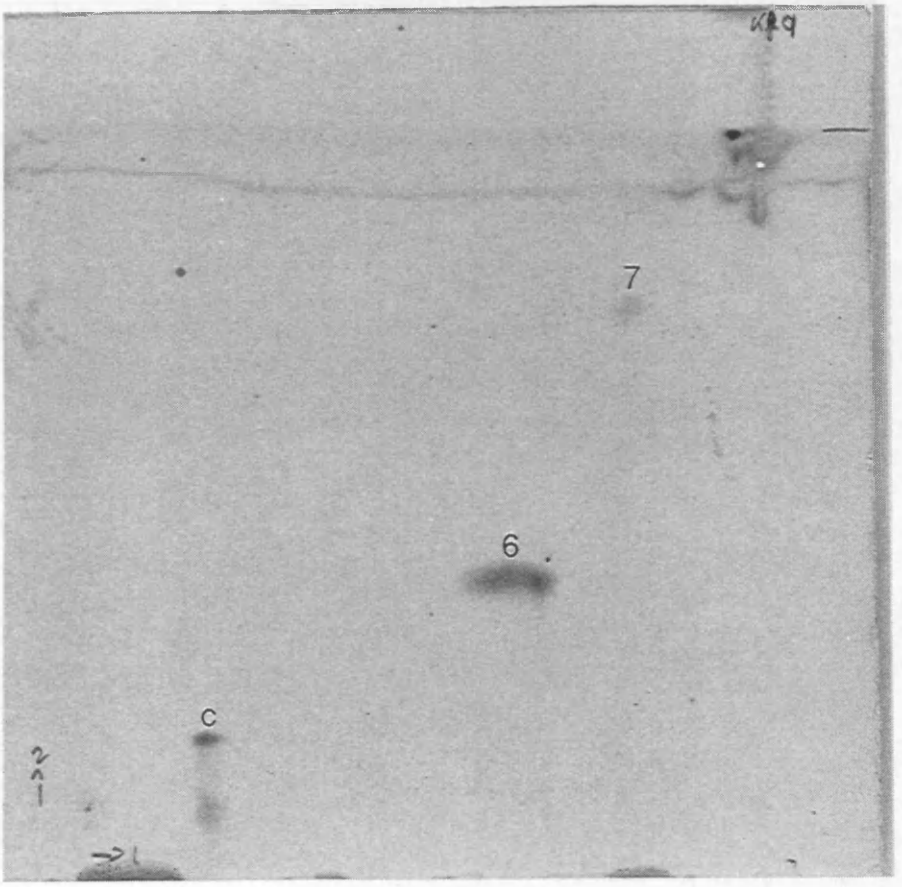
Figure 3.13 (a) Cells grown at 30°C.



Key
6. Glycolipid (purple-blue).
7. Glycolipid (purple-blue).
c. Free sugar [See Section 3.1]
d. Non- sugar containing polar lipid (Phosphatidylglycerol) as spot is brown, rather than purple-blue [See Section 3.1].
Arrows on the bottom left-hand corner of the plate indicates the direction of the 1st and 2nd phase.

Figure 3.13 Identification of a polar lipids containing sugar moieties (using α-naphthol stain). Spots staining purple-blue were identified as glycolipids and other polar lipids present on the plates, do not contain sugar moieties as they are the incorrect colour (brown).

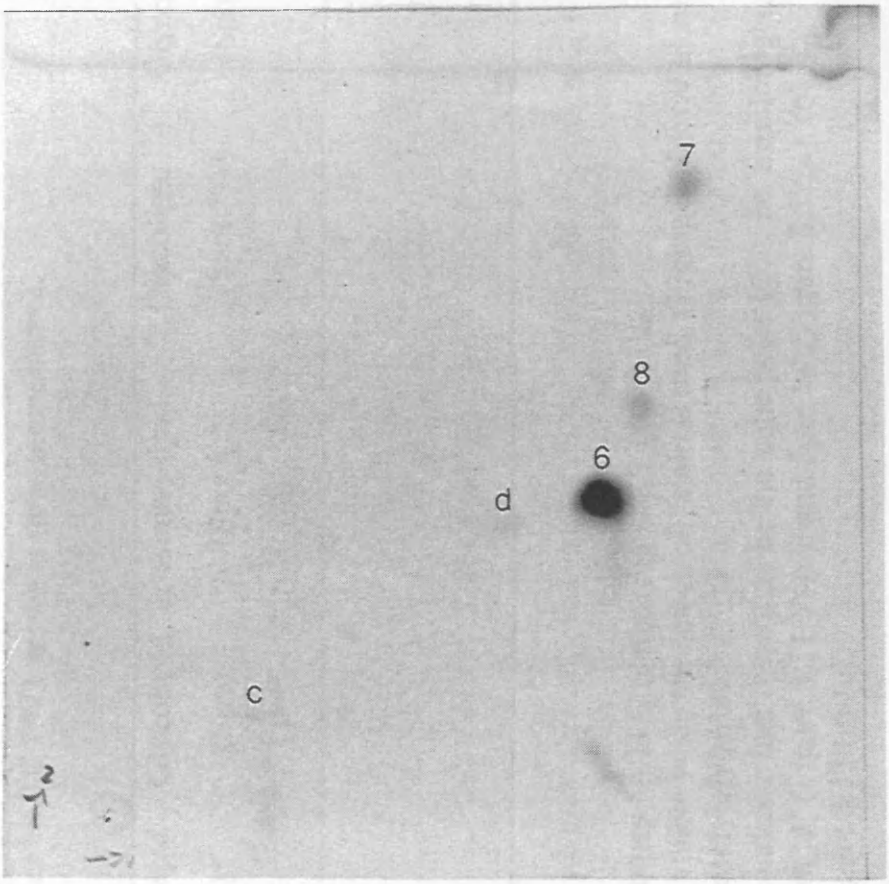
Figure 3.13 (b) Cells grown at 20°C.



Key
6. Glycolipid (purple-blue).
7. Glycolipid (purple-blue).
c. Free sugar [See Section 3.1]
Arrows on the bottom left-hand corner of the plate indicates the direction of the 1st and 2nd phase.

Figure 3.13 Identification of a polar lipids containing sugar moieties (using α-naphthol stain). Spots staining purple-blue were identified as glycolipids and other polar lipids present on the plates, do not contain sugar moieties as they are the incorrect colour (brown).

Figure 3.13 (c) Cells grown at 10°C.



Key
6. Glycolipid (purple-blue).
7. Glycolipid (purple-blue).
8. Glycolipid (purple-blue).
c. Free sugar [See Section 3.1]
d. Non- sugar containing polar lipid (Phosphatidylglycerol) as spot is brown, rather than purple-blue [See Section 3.1].
Arrows on the bottom left-hand corner of the plate indicates the direction of the 1st and 2nd phase.

Table 3.5 Polar lipids of *L. monocytogenes* NCTC 7973 grown at three temperatures.

Temperature	**PG**	**DPG**	**Phospholipid (Spot 2)***	**Glycolipid (Spot 1)**[a]**	Amino-lipid (Spot 4)[a]	Glycolipid (Spot 7)	Glycolipid (Spot 8)
30°C	+	+	+	+	+	+	-
20°C	+	+	+	+	+	+	-
10°C	+	+	+	+	+	+	+

+ Present. - Absent.
Numbers in parentheses indicate spot numbers on TLC plates (Figures 3.10-3.13).
Bold characters indicate polar lipid spots visible when the 'catch all' stain is used, (Figure 3.10), for identification see text.
PG Phosphatidylglycerol. DPG Diphosphatidylglycerol.
[a]The position of the individual lipid spots indicate that these may be the same polar lipid, i.e. a amino-glycolipid [See text].
*This spot was also variously labelled Spot '3' (Figure 3.11(a)-(c)) and Spot 'a' (Figure 3.12(a), (b).
**This spot was also labelled spot '6' (Figure 3.13 (a)-(c).

3.4 THE UPTAKE AND UTILISATION OF DIFFERENT CARBOHYDRATES BY *L. monocytogenes*

Experiments were performed to investigate the possibility of growth temperature influencing the ability of *L. monocytogenes* to utilise different carbohydrates. Initially 20 carbohydrates were studied at two growth temperatures, 10°C and 30°C, and in order to screen rapidly, growth was judged by eye on a no-growth, growth, good growth basis. Any carbohydrate supporting growth at a single growth temperature only was tested a second time, before being studied in more detail and growth rate on that carbohydrate determined. The results of these experiments are given in Table 3.6 and indicate that of the 20 carbohydrates examined, glucose, α-L-rhamnose, D-fructose, D-cellobiose, D-mannose, α-methyl-D-glucoside, and α-methyl-D-mannoside were able to support growth at both the study temperatures.

Three of the carbohydrates tested were able to supported the growth of *L. monocytogenes* at a single temperature only, these were: lactose, (positive at 30°C only); and D-mannitol, and α+xylose (positive at 10°C only). However when the experiment was repeated, none of the three carbohydrates were able to support the growth of *L. monocytogenes* at either growth temperature.

This inability of any of the carbohydrates to sustain growth at one temperature only, meant that any studies investigating any differences in carbohydrate uptake at a particular growth temperature could not be done. Consequentially, studies looking at differences in the rate of uptake of glucose at a range of growth temperatures were performed.

Table 3.6 Ability of a range of carbohydrates to support the growth of *L. monocytogenes* at 10°C and 30°C.

Carbohydrate	30°C	10°C
α-L-Rhamnose	+	+
D-Fructose	+	+
D-Maltose	+/-[1]	+/-[1]
D-Cellobiose	+	+
D-Mannitol	-	+/-*
D-Mannose	+	+
α-methyl-D-Glucoside	+	+
D-Lactose	+/-*	-
D-Galactose	-	-
D-Ribose	-	-
D-Arabinose	-	-
Dextrin	-	-
D-Glycogen	+/-[1]	+/-[1]
Sucrose	-	-
D-Sorbitol	-	-
α-methyl-D-Mannoside	+	+
D-Sorbose	-	-
α-D-Melibiose	-	-
α+Xylose	-	+/-*
D-Glucose	+	+
No carbohydrate	-	-

+ good growth, +/- growth, - no growth, [1]very poor growth; *positive first time, negative upon repeating.

3.5 USE OF MEMBRANE VESICLES TO STUDY GLUCOSE UPTAKE

Membrane vesicles have been used previously to study glucose uptake in bacteria (Kaback, 1971; Buckley and Hamilton, 1994), however before any study using *L. monocytogenes* could be made, time had to be spent maximising the production of the vesicles and also ensuring that a suitable technique was developed to accurately quantify the amount of vesicles produced. In order to prevent any change in the membrane characteristics after growth at a particular temperature, the preparations were maintained at this temperature at all times until the vesicles were formed. This presented problems as the method for producing membrane vesicles used a number of enzymes: namely lysozyme, DNase and RNase, each with an optimum temperature. Therefore correct incubation times for these enzymes at each growth temperature had to be found.

Initial studies, concentrated on the production of membrane vesicles from cells grown at 30°C in TM medium under batch conditions. These studies focused on three factors; the ability of the method used to produce membrane vesicles from *L. monocytogenes*, the accuracy of the quantitation procedures, and the reproducibility of the results. Figures 3.14 and 3.15 shows the presence, using transmission electron micrographs, of membrane vesicles formed from whole cells of *L. monocytogenes*. The vesicles were a range of diameters between 0.5 and 2μm. The publication that formed the basis of the procedure used here (Kaback, 1971) indicated that the final vesicle preparation should not have in excess of 0.05% of the initial numbers of viable organisms, and the final protein concentration should be 4-7mg/ml. The typical amount of viable cells present in the vesicle preparation was well within the parameter set by Kaback, (1971), (Initial:Final CFU/ml, $5.8 \times 10^9 : 2.62 \times 10^3$) and whilst the same vesicle preparation gave a protein concentration of 0.15mg/ml, lower than the figure given by Kaback (1971), this value could have been increased by significantly

reducing the amount of buffer used to resuspend the vesicles. This could be done without adversely effecting the purity of the sample.

The results of the vesicle preparation (Figures 3.14 and 3.15) show that the production of membrane vesicles from whole cells of *L. monocytogenes* is possible, and therefore (in the presence of a suitable electron donor) glucose uptake studies could be performed. However the problem of accurately quantifying the actual number of vesicles produced remained. The final CFU/ml value indicated low quantifiable numbers of whole cells present, but this is not a measure for vesicles, nor any cell debris present; the protein assay accurately estimates the total protein, but not the total amount of protein present in the vesicles only.

Therefore in order to accurately compare the amount of glucose uptake by cells grown at different temperatures the amount of vesicles present has to be known. It is possible that if a single method was used for each set of cells (i.e.. cells grown at different temperatures) that the amount of vesicles, residual cells, and cell fragments could be, with care, reproduced. However, this would mean any cell-enzyme incubations would have to proceed at a single temperature and for a set time period, thereby causing a possible temperature mediated change in the composition of the cell membrane. This problem of reproducibility when processing different sets of cells, was sufficient for an alternative method of measuring glucose uptake using whole cells to be employed.

Figure 3.14 Negatively stained electron micrographs showing membrane vesicles from whole cells of *L. monocytogenes* using the method of Kaback, (1971). (Magnification: x50000).

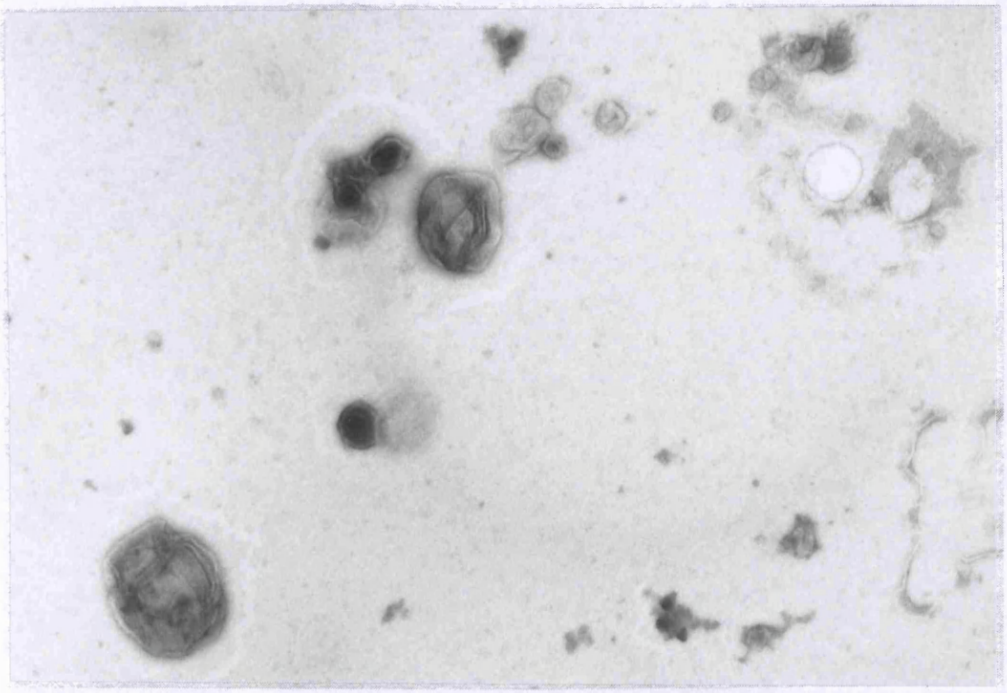
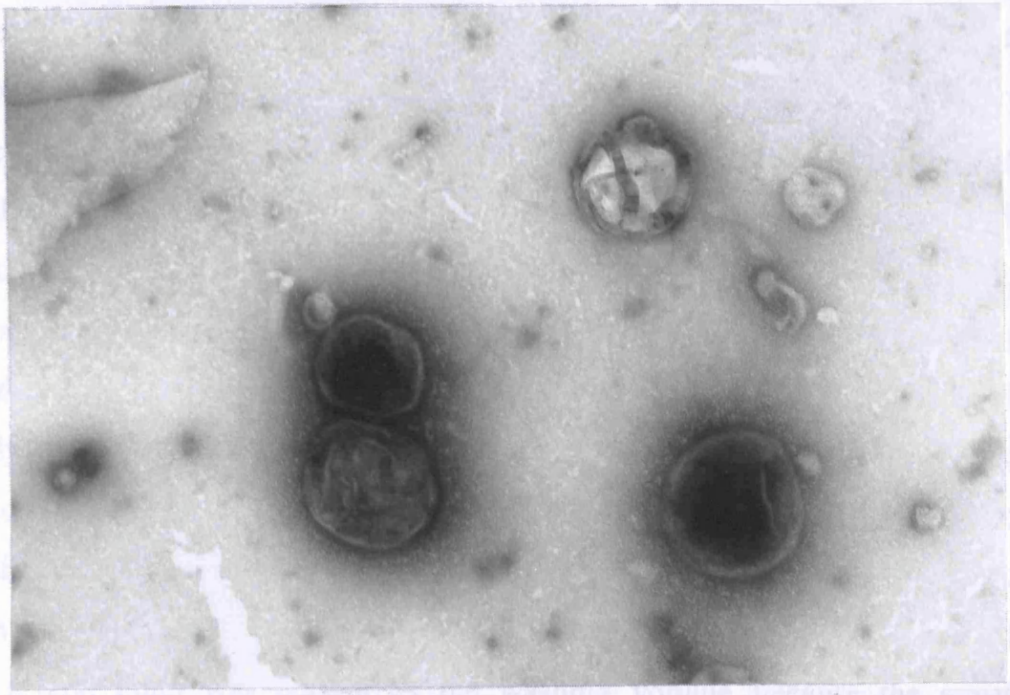


Figure 3.15 Negatively stained electron micrographs showing membrane vesicles from whole cells of *L. monocytogenes* using the method of Kaback, (1971). (Magnification: x30000).



3.6 USE OF WHOLE CELLS TO STUDY GLUCOSE UPTAKE

Glucose uptake by *L. monocytogenes* has been measured before using whole cells and a radiolabelled non-metaboliseable analogue of glucose, 2-deoxy-D-glucose (Wilkins *et al.*, 1972). However as the method used here was a modification of that employed by Herbert and Bell, (1977) to study glucose uptake, time was spent optimising the procedure. This concentrated on finding the a suitable membrane type and filtration assembly for capturing and washing cells. However, it was most important to ensure that any radiolabelled 2-deoxy-D-glucose not associated with the cells was removed from the filters during the washing stage. The washing stage was a key stage in minimising background and maximising reproducibility. Furthermore in order to optimise the procedure, the amount of water required to adequately wash the cells, and the amount of residual radiolabelled 2-deoxy-D-glucose present on the membranes were both analysed. In order to assess the amount of water required to remove incorporated radiolabelled 2-deoxy-D-glucose, a range of volumes of water were tested in the washing procedure (Table 3.8). This study showed that increasing wash volumes above 10ml did not increase the amount of free 2-deoxy-D-glucose removed from the filters. 20ml was chosen as the washing volume because the apparatus employed to capture the cells onto a membrane (Buchner filtration assembly) had a relatively large diameter (47mm), and, if the vacuum was too strong there was a distinct possibility that if too small a volume, the water would be sucked through the assembly before coming into contact with all of the filter. The average value for background counts were found to be 19.6CPM.

Table 3.7 Effect of removal of free radiolabelled 2-deoxy-D-glucose from whole cells of *L. monocytogenes*.

Volume of washing water (ml)	0	10	20	40	60
CPM	4102	217	243	280	276

CPM values indicate the residual radioactivity remaining on filter membranes after washing. Incubation time 2 mins. Mean of duplicate samples.

Table 3.8 Uptake of 2-deoxy-D-glucose by *L. monocytogenes* incubated on ice and at 30°C. Cells used were grown in the chemostat at 30°C.

Time (hr)\ Assay Temp.	0°C[1]	30°C
0.5	199	244
1	218	443
2	272	529

[1]Cells assayed on ice. All values are for CPM. (CPM values indicate the residual radioactivity remaining on filter membranes after washing).

3.6.0 Uptake of 2-deoxy-D-glucose by *L. monocytogenes*

Uptake of 2-deoxy-D-glucose by *L. monocytogenes* was analysed using five replicates at each incubation temperature [See Appendix III].

Two phases of association of 2-deoxy-D-glucose with *L. monocytogenes* were seen. An average of 4.95 μMoles 2-deoxy-D-glucose mg^{-1} cells (248 CPM) [See Appendix III] were associated with cells within 15 sec of incubation (shown as time = 0) (Figures 3.15-17). This rapid association was seen at a similar extent whatever the growth or assay temperature. Cells grown in the chemostat at 30°C, but maintained on ice, also showed similar levels of association after 0.5, 1, and 2 hours, whilst those assayed at 30°C continued to take up 2-deoxy-D-glucose over the same period (Table 3.8). It is possible therefore that the initial rapid uptake values seen at all growth / assay temperatures (Figures 3.15-17) are for residual radiolabelled 2-deoxy-D-glucose present on the filter membranes after the washing stage.

3.6.1 Cells grown at 30°C and 10°C

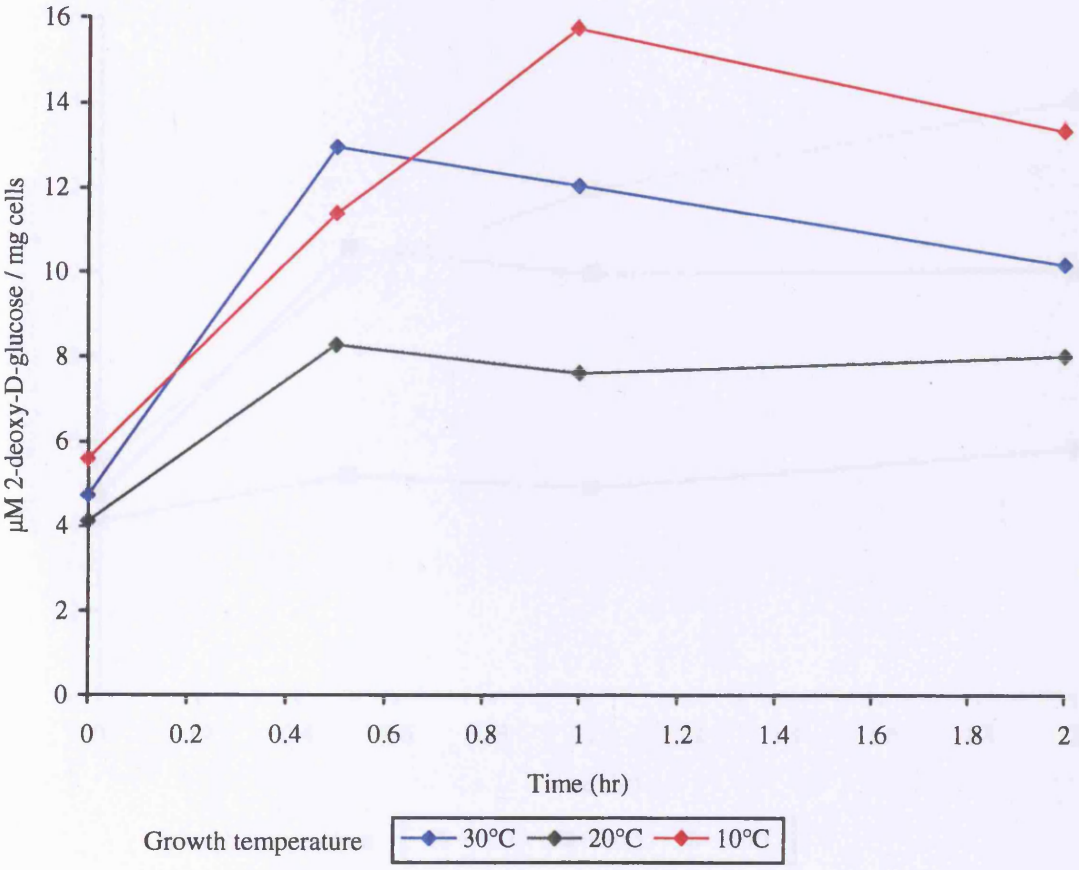
After the initial rapid association cells grown at 30°C or 10°C continued to take up 2-deoxy-D-glucose, when uptake was assayed at 30°C, 20°C, or 10°C (Fig 3.15-3.17), whilst, over the same period, cells maintained on ice took up minimal amounts of 2-deoxy-D-glucose (Table 3.8). Not suprisingly the temperature at which uptake of 2-deoxy-D-glucose was measured had a significant effect ($P<0.05$) on the rate of uptake. Cells assayed at 30°C (Fig 3.15) had a faster rate of uptake than cells assayed at 20°C (Fig 3.16), which in turn were faster than cells assayed at 10°C (Fig 3.17). Cells grown at 10°C were able to take up 2-deoxy-D-glucose faster than cells grown at 30°C when assayed at 30°C ($P<0.05$). However, when the cells were assayed at 20°C and 30°C there was no significant influence ($P>0.05$) on uptake of 2-deoxy-D-glucose from the temperature at which cells were grown prior to measurement of uptake. Thus cells grown at

10°C were more able to take up 2-deoxy-D-glucose at 30°C, but not at 20°C or 30°C, than cells grown at 30°C.

3.6.2 Cells grown at 20°C

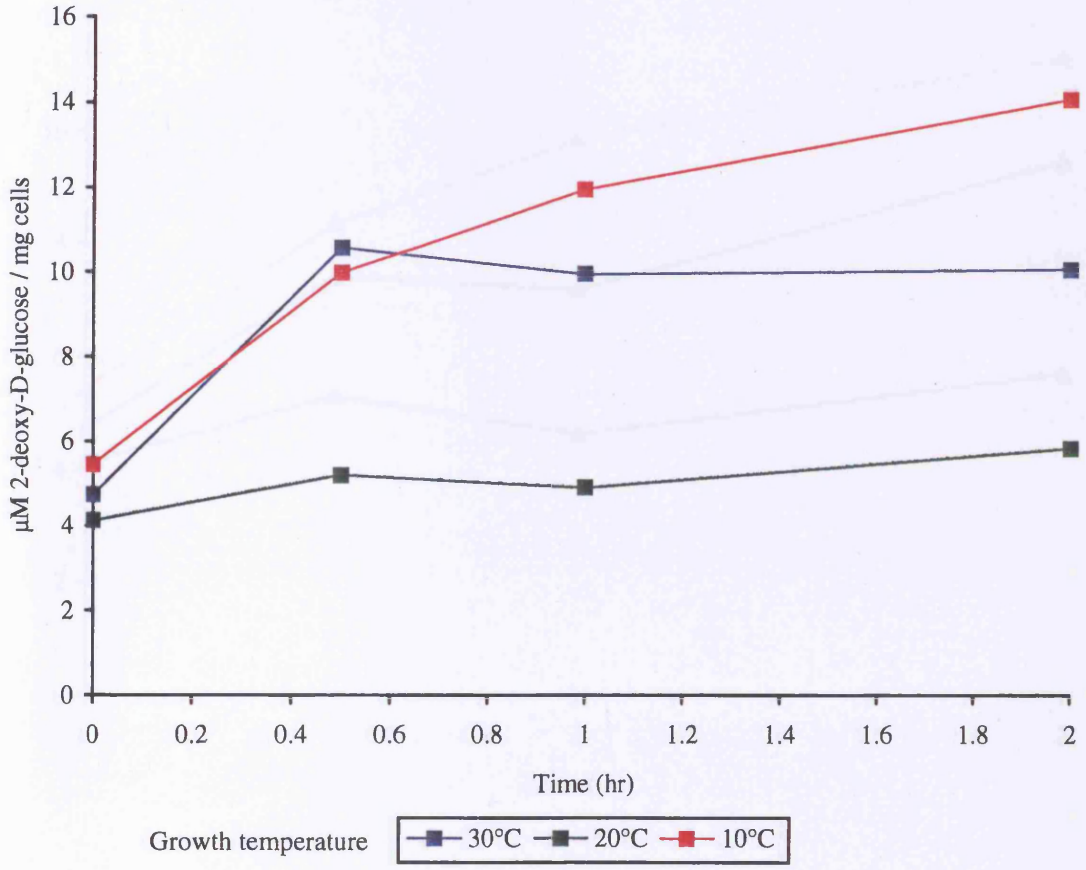
The cells grown at 20°C gave consistently lower uptake values than the cells grown at the other two temperatures, irrespective of the assay temperature (Figures 3.15-3.18). If the raw data are scrutinised it is clear that the standard error values are generally lower than the values for other growth temperatures [See Appendix III]. Therefore the lower values seen for cells grown at 20°C are not due to a set of outlying values. The lower values seen are not easily explained but, possibly due to a fault within the experiment using the 20°C grown cells only, either because a portion of the cells used in the experimental were in some way compromised and unable to take up 2-deoxy-D-glucose, or by an incorrect amount of radiolabel being added. Despite the problems with this experiment, analysis of the values gained (Figure 3.18) does show some assay temperature mediated response with raised uptake values ($p<0.05$) seen at an assay temperature of 30°C, compared with uptake at the other two temperatures.

Figure 3.15 The effect of growth temperature on the rate of 2-deoxy-D-glucose assimilation by *L. monocytogenes* NCTC 7973. Figure shows the rate of uptake assayed at 30°C.



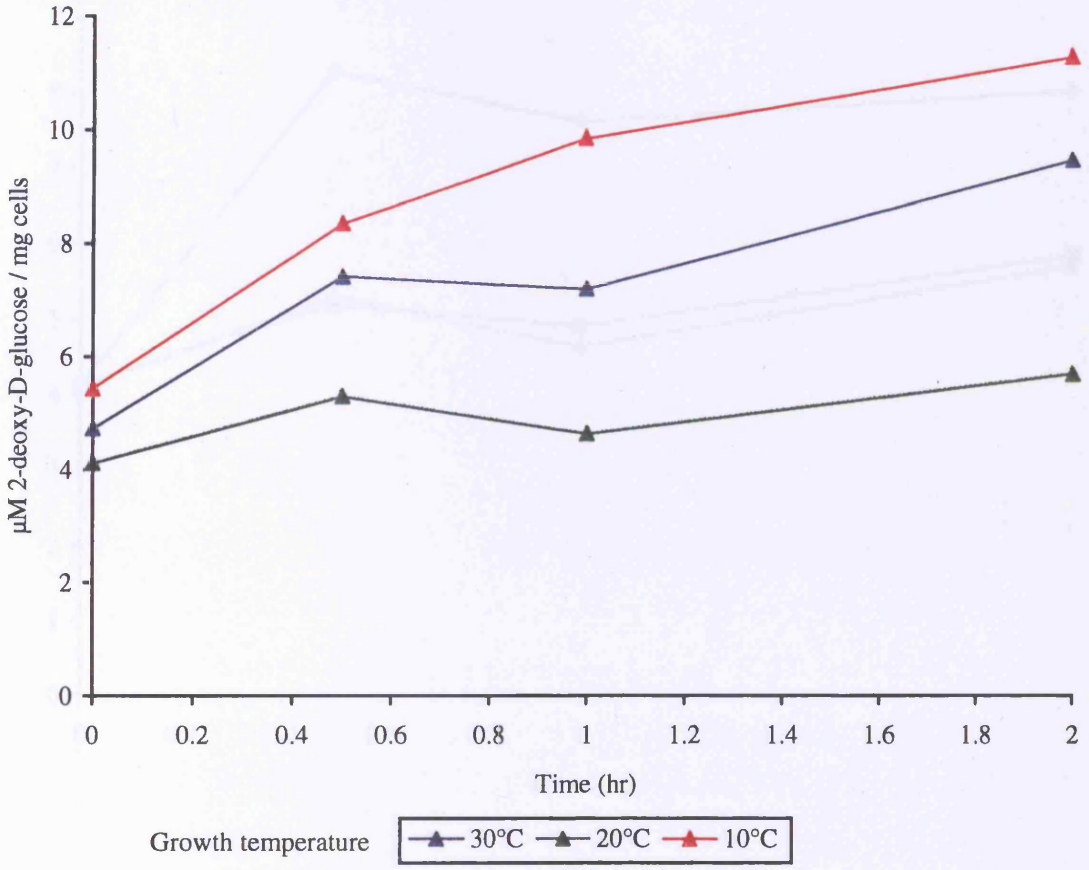
Cells grown in the chemostat using TM medium (Trivett and Meyer, 1971) under carbon limitation, at three different growth temperatures. Initial sample (Time = 0) taken after 15 seconds.

Figure 3.16 The effect of growth temperature on the rate of 2-deoxy-D-glucose assimilation by *L. monocytogenes* NCTC 7973. Figure shows the rate of uptake assayed at 20°C.



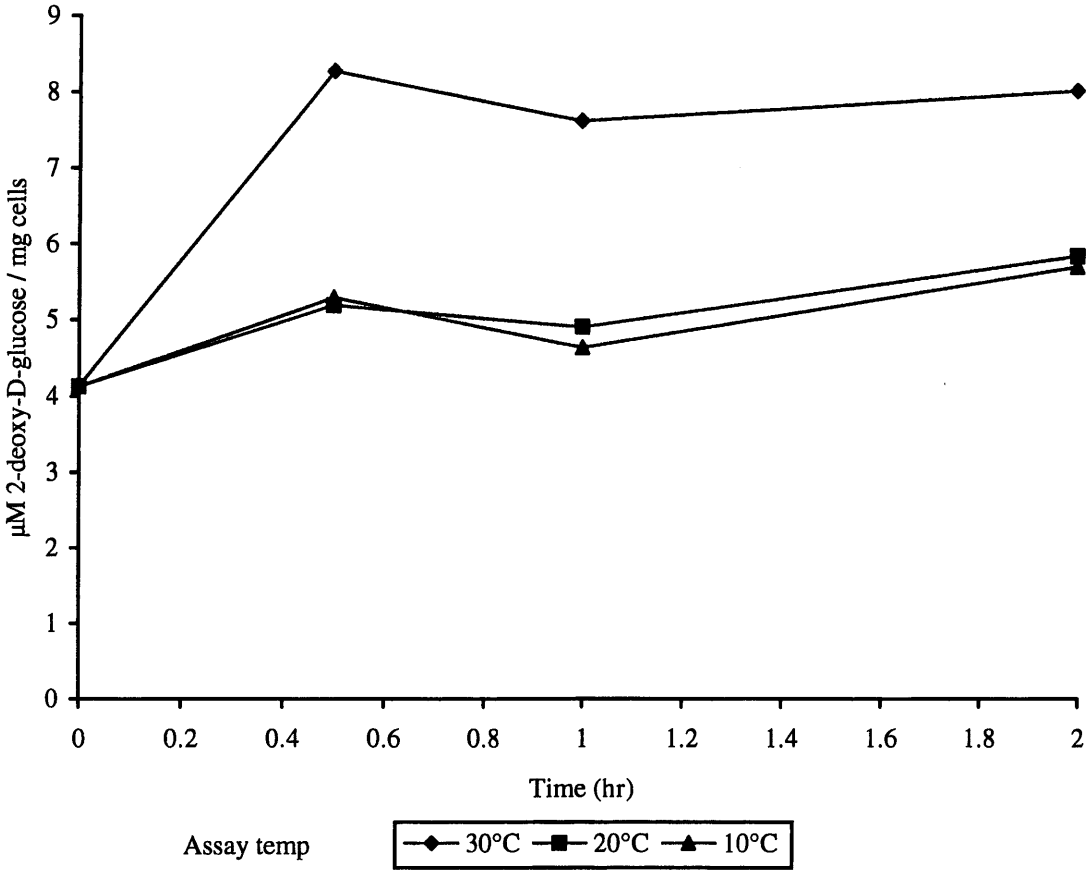
Cells grown in the chemostat using TM medium (Trivett and Meyer, 1971) under carbon limitation, at three different growth temperatures. Initial sample (Time = 0) taken after 15 seconds.

Figure 3.17 The effect of growth temperature on the rate of 2-deoxy-D-glucose assimilation by *L. monocytogenes* NCTC 7973. Figure shows the rate of uptake assayed at 10°C.



Cells grown in the chemostat using TM medium (Trivett and Meyer, 1971) under carbon limitation, at three different growth temperatures. Initial sample (Time = 0) taken after 15 seconds.

Figure 3.18 The effect of assay temperature on the assimilation of 2-deoxy-D-glucose by the cells of *L. monocytogenes* NCTC 7973 grown at 20°C.



Cells grown in the chemostat using TM medium (Trivett and Meyer, 1971) under carbon limitation, assayed at three different growth temperatures.

Initial sample (Time = 0) taken after 15 seconds.

4. DISCUSSION

4.1 THE CONTINUOUS CULTURE OF *L. monocytogenes* USING A DEFINED MEDIUM

4.1.0 Comparison of the six defined media for *Listeria monocytogenes*

The importance of a finding a suitable defined medium that supported growth of *L. monocytogenes* at a range of temperatures between 30°C and 10°C in a chemostat, before any lipid and carbohydrate analyses could be attempted, cannot be overstated. Within complex media there are intrinsic variations in the precise chemical composition and this may effect any solute uptake and lipid analyses made. Also, there are reports of organisms being able to incorporate lipids present in some components of complex media e.g. yeast extract, plant extracts, peptones (Ratledge and Wilkinson, 1988). The use of a defined medium, free from any lipid-containing components removes any possibility of the fatty acid and lipid composition of *L. monocytogenes* being effected directly by the growth medium. Also, as the defined medium will have a single carbon source (glucose), uptake rates can be accurately assessed.

Several defined media had been described for *L. monocytogenes,* but they had not been compared, nor had their ability to support the growth of the organism in continuous culture been tested. Therefore the first part of this project sought to find the most suitable of the published defined media for the growth at 30°C of *L. monocytogenes,* NCTC 7973, the strain chosen for all subsequent studies, and also two further strains. Once the most suitable medium was found, further studies were performed to investigate the anaerobic growth of the chosen strain, and also more importantly, its ability to support continuous culture of *L. monocytogenes* NCTC 7973 at a range of temperatures between 10 and 30°C.

The results of the study showed that, the defined medium described by Trivett and Meyer, (1971) was most suitable for use in continuous culture not only because of its good overall performance in terms of yield and growth rate for

each of the strains tested, but also because its composition was fairly simple, with only 12 heat labile components. Of the other defined media tested, only two had a similar or fewer number of heat labile components (Premaratne *et al.*, 1991, and Ralovich *et al.*, 1977), and both of these gave less favourable growth results. Paramount consideration must be given to the composition of the medium used for continuous culture because relatively large amounts of the medium are prepared, (two separate aspirators containing 10 litres) and though many of the medium components are able to withstand autoclave sterilisation, the heat labile components have to be filtered and pumped into the aspirators aseptically. Furthermore, cost of materials can be a factor in choosing a medium to be used in large volumes. This was not, however a decision criteria here.

Of the six defined media for *L. monocytogenes* studied, five were able to support acceptable sequential growth of each of the different strains, except in one instance. Strain NCTC 5105 failed to grow in the medium of Welshimer (1963) beyond the second transfer (Table 3.1). The sixth medium (Siddiqi and Khan, 1989), was only able to support growth of the organism when inoculated with an overnight culture of any of the three strains grown on solidified Welshimer medium (Welshimer, 1963) at 30°C.

The inability of the media of Welshimer, (1963) and Siddiqi and Khan, (1989) to support sequential growth of strain NCTC 5105, and all three strains respectively, deserves further comment. Welshimer, (1963) did not use strain NCTC 5105, but demonstrated growth of a range of strains, including one with the same serotype (3). As Welshimer's medium was of a fairly simple composition (3 salts, glucose, 9 amino acids, 4 vitamins) it is possible that the sequential growth of NCTC 5105 was unable proceed due to absence of an essential component present in the other media. NCTC 5105 grew sequentially in the medium of Trivett and Meyer, (1971) which used the same vitamin composition as Welshimer, (1963), and also sequential growth was seen in the

medium of Ralovich (1977) which only contained four amino acids, each present in Welshimer's medium. Therefore the inability of strain NCTC 5105 to grow in Welshimer's medium is unlikely to be attributable to the lack of a vitamin or amino acid. The medium of Welshimer, (1963) did not however, contain an iron component. Iron has been shown to be stimulatory for growth of *L. monocytogenes,* particularly if the culture is aerated (Sword, 1966; Trivett and Meyer, 1971). Of the three strains tested, NCTC 5105 grew poorest in each of the other four defined media (Table 3.1) and, as each of these media contained an iron source, it is possible that the lack of iron in Welshimer's medium was a vital factor in its inability to support the strain's growth. In considering the absence of iron in the medium of Welshimer, (1963) it is of interest to look at work of Trivett and Meyer, (1971). For this study they used the medium of Welshimer, (1963) as an 'iron free inoculum' for growing strain A4413, a strain also used by Welshimer, (1963). When cells grown in this way were inoculated into their defined medium Trivett and Meyer, (1971) showed that growth was reduced by two-thirds in the absence of iron. Therefore it is possible there was an initial 'carry-over' of iron from the complex medium-grown stock cultures of strain A4413, that could enhance its growth in the Welshimer medium.

The results of the study presented here show growth of strain NCTC 5105 on solidified Welshimer medium, and upon initial subculture to the same medium without agar. It is possible that there were traces of iron carried over into both of these cultures from the stock culture which was kept on tryptose soy agar, and by the second subculture, the amount of iron available was reduced to a level that did not facilitate the growth of the strain.

Despite Siddiqi and Khan, (1989) showing their medium able to support the growth of a range of *L. monocytogenes* strains including NCTC 7973, it, and the other two strains were unable to grow beyond the initial subculture in the results described here. The composition of the defined medium of Siddiqi and Khan,

(1989) was similar to others used in the study, with notable exceptions, the bases adenine, cytosine, guanine and thymine were present, and also the vitamin, thioctic acid, shown by Welshimer (1963), but not Ralovich *et al.*, (1977) to be essential for the growth of *L. monocytogenes* was not present. In addition, the amino acids arginine, histidine, and cysteine, each present in the medium of Trivett and Meyer (1971) were absent. It is possible that the lack of one (or more) of these medium components prevented the sequential growth of the organism in the medium in the present study. Siddiqi and Khan, (1989) demonstrated that their medium was able to support the growth of strain NCTC 7973, though the methods used in their study to eliminate nutrient 'carry over' from the original stock cultures were not as fastidious as those used here, and this may have been a contributing factor in their ability to demonstrate growth. It may also explain why growth was seen upon initial subculture from solidified Welshimer medium into the medium here.

The inability of the medium of Welshimer, (1963) and Siddiqi and Khan, (1989) to support the sequential growth of one or all the strains of *L. monocytogenes* was also noted by the authors of another of the defined medium tested (Premaratne *et al.*, 1991), The same authors also noted a similar inability to support sequential growth in two other published defined media for *L. monocytogenes;* those by Friedman and Roessler, (1961); and Trivett and Meyer, (1971). This study however, shows that each of these media is able to support sequential growth of the three strains tested (Table 3.1). Indeed these two media also gave consistently the best values for growth rate or cell yield. It is unclear why Premaratne *et al.*, (1991) was unable to demonstrate sequential growth using these two media, however none of the strains used here were used in their work, though some of the strains did have the same serotypes (1, 4b).

In a wide ranging nutritional study, leading to the developing of a defined medium for strains of *L. monocytogenes,* Ralovich *et al.*, (1977) concluded that

there were no constant links between the nutritional requirements (of amino acids and vitamins), and serotype, agglutinability, haemolytic properties or virulence in strains of the organism. Therefore although there may be variability in the nutritional requirements of individual strains that accounts for inability of Premaratne *et al.*, (1991) to maintain sequential growth in these two defined media, at the present time the cause or correlating parameter is unidentified. Premaratne *et al.*, (1991) indicated that, when compared to other media, their defined medium was the most suitable for the growth of *L. monocytogenes.* However in this study, despite giving the greatest value for yield, and close to the highest value for the maximal growth for the culture of *L. monocytogenes* strain NCTC 4885, it gave very poor results for the other two strains tested and therefore was not considered for use.

The factor determining the growth yield in batch culture is not clear. The concentration of the carbon source is one possibility but neither the growth rate nor yield under either batch culture condition (Table 3.1 and 3.2) were related to the amount of glucose present in the media. The best overall medium (Trivett and Meyer, 1971) contained less glucose (2g l^{-1}) than any of the other media compared.

Whilst considering the results of the defined medium comparison, it is important to bear in mind the primary reason for the study, i.e. to find the most suitable for the growth of *L. monocytogenes* NCTC 7973 in a chemostat at a range of temperatures. The chosen medium of Trivett and Meyer, 1971 proved to be adequate for this purpose. A fuller comparison of the chosen media, using the same method as described, but involving more strains, including those used by other workers, may go some of the way to explaining the discrepancies between the results seen here and those by Premaratne *et al.*, (1991), and would possibly give a single overall defined medium, suitable for the entire species, *L. monocytogenes.*

4.1.1 Continuous culture of *L. monocytogenes*

The continuous culture of *L. monocytogenes* using a defined medium was fundamental to the success of the project. A failure to achieve continuous culture, with changes in growth temperature being the single variable parameter, would prevent any responses seen by the organism to be directly correlated to temperature.

The continuous culture of *L. monocytogenes* at growth temperatures between 10 and 30°C with a defined medium had not been used in any previously published work. As a result time had to be spent designing a suitable chemostat system. The problems overcome prior to achieving continuous culture are discussed below [See 4.1.2].

All problems related to the growth of *L. monocytogenes* in a chemostat were overcome. Figures 3.2 and 3.3 indicate how D_c values for continuously cultured cells of *L. monocytogenes* using the defined medium of Trivett and Meyer, (1971) were obtained, with values of $0.22h^{-1}$ and $0.025h^{-1}$ seen for cells grown at 30 and 10°C respectively. The same graphs also indicate that the limiting nutrient at both temperatures is glucose.

4.1.2 Problems during the continuous culture of *L. monocytogenes*

There were several problems encountered whilst obtaining the successful continuous culture of *L. monocytogenes.* Initial problems concerned contamination, and this occurred most commonly while trying to sterilise the large volumes of defined medium that were required. The methods used to sterilise the medium, in particular the heat labile components [See 2.3.2], possibly tended towards overcaution. However, the persistent inability to achieve the sterile addition of the 150ml concentrate, containing the heat labile components, was the principle source of chemostat contamination and was only

overcome by repeated 0.2μm filter-sterilisation steps. In view of the time periods lost to contamination before this method of sterilisation was employed its use was continued.

A further problem occurred whilst growing the cells at 10°C. It was found that in order for the successful continuous culture of *L. monocytogenes* at 10°C, the temperature had to be reduced stepwise (in 1°C increments) from 15°C. If growth at 10°C was attempted without this 'conditioning' stage, washout occurred before steady state was achieved. It is possible that this gradual reduction in growth temperature enabled similar physiological adaptation(s) to those seen by Walker *et al.*, (1990) and Buchanan and Klawitter, (1991). These studies indicated that a pre-incubation step at a low temperature reduced the duration of lag phase during the growth of *L. monocytogenes* at low temperatures. Walker *et al.*, (1990) postulated that pre-incubation at a low temperature reduced a 'cold shock' effect (Ingram and Mackey, 1976) which is caused by both a reduction in growth temperature and also the rate of cooling. Thus the 'stepwise' reduction in cells grown in the chemostat could be important in minimising cold shock.

4.1.3 Cell lysis during batch and continuous culture of *L. monocytogenes*

In addition to the difficulties described above, significant problems were also encountered with cell lysis within the chemostat. Cell lysis was a major problem and occurred in two separate forms.

Rapid and irreversible cell lysis was seen during the late exponential / early stationary growth phase during the anaerobic growth of *L. monocytogenes* in the medium of Trivett and Meyer (1971) (Figure 3.1). Reports of lysis at a similar phase of growth have been noted for a range of strains of *L. monocytogenes* (including NCTC 7973) grown anaerobically and aerobically (Trivett and Meyer, 1967; Tyrrell, 1973; Pine *et al.*, 1989).

A rapid and irreversible cell lysis also was seen in the chemostat soon after inoculation (Figure 3.4), though lysis was not observed during the aerobic batch study of six defined media. During the period directly after inoculation of the chemostat, the cells were grown under batch conditions (i.e. with the medium pump off) in order to build up bacterial cell mass. Towards the end of exponential phase of growth, at an OD_{600nm} of approximately 2.0, lysis occurred. Given that both the instances of lysis described here occurred during a period of batch culture at similar growth phases, lysis could be due to a common cause triggered in, or acting upon *L. monocytogenes* during the early stationary growth phase of cells grown either aerobically or anaerobically.

Trivett and Meyer, (1967) showed rapid lysis in *L. monocytogenes* after 24h with cells grown at 37°C without shaking, in trypticase broth, supplemented with glucose. These workers attributed the lysis to a catastrophic reduction in pH. However work by Tyrrell (1973) showing autolysis in a range of strains of *L. monocytogenes* (including NCTC 7973) after 24-48h growth at 35°C in brain-heart infusion broth, with shaking, showed that lysis occurred independently of pH, and these workers speculated that the effect was caused by cell wall degradation related to a previously noted instability in isolated cell walls of *L. monocytogenes* (Tinelli, 1965, 1969). It is unlikely that either of the instances of autolysis seen in the results presented here, is due to a reduction in pH. The pH of the medium containing lysed anaerobically grown cells was 7.03: a value considered conducive for near-maximal growth of the organism, and well above minimum growth pH values (Seeliger and Jones, 1986; George *et al.*, 1988; Farber *et al.*, 1989). The cells that lysed in aerated conditions were grown in the chemostat, equipped with a pH control, which prevented the pH from falling below 6.8 [See 2.2.0]. As there was no sudden significant reduction in pH values in either case, there seems little possibility that pH was a contributing factor in the onset of cell lysis.

Pine *et al.*, (1989) noted lysis in a range of *L. monocytogenes* strains grown in defined and semi-defined medium with glucose as the sole carbon source. In both cases the lytic effect was most pronounced under anaerobic conditions, and was not due to a decrease in pH. The use of glucose as the carbon source however, appeared to exacerbate the problem. Alternative carbon sources, such as galactose, rhamnose, xylose and lactose caused very little or no lysis; and growth at lower temperatures also appeared to lessen significantly any lytic effect (Pine *et al.*, 1989).

The effect of a decreased growth temperature on cell lysis in anaerobic batch culture was not studied here, but cell lysis in the chemostat was observed only at a growth temperature of 30°C. Of the four carbohydrates shown by Pine *et al.*, (1989) to decrease the effect of lysis, growth was only demonstrated using rhamnose by strain used here, (NCTC 7973) [See 3.4]. Therefore, an alternative solution to the one used may have been to use rhamnose as a carbon source. However, it was found that by initially simulating 'fed-batch' conditions and closely monitoring the OD_{600nm} as described in the results section, the problem of lysis could be avoided and this was the solution employed.

Pine *et al.*, (1989) suggested that lysis was a consequence of the carbon source used, but as they did not perform additional experiments to investigate the cause of the cell lysis, they noted that the lysis could also have been due to phage, production of an autolytic enzyme or unbalanced growth and aberrant cell wall formation. As the main purpose of the work presented here was not an investigation of autolysis in *L. monocytogenes* and the measures described in the results prevented a re-occurrence of the phenomenon, no work to investigate the source of either the aerobic nor the anaerobic lysis was performed, except that the presence of phage was checked in both the medium supernatant and the residual pelleted cells by J.McLauchlin, PHLS, Colindale, London, UK., and none were found.

It of interest to speculate as to the cause of the lysis, however. Weunscher *et al.*, (1993) showed that a major extracellular protein of *L. monocytogenes*, p60, is a murein hydrolase, and as it is, by definition, listerial autolysin. Autolysins are important in cellular processes such as cell wall growth, turnover and splitting of the septum for cell separation (Holtje and Tuomanen, 1991). Protein p60 was shown to be present in raised amounts during the stationary, compared to the exponential period of growth of *L. monocytogenes*, and caused a shortening of bacterial chains (Weunscher *et al.*, 1993). The same authors looked for autolysis in each of their strains by growing them for an extended time period in brain heart infusion (BHI) broth. However they were unable to detect lysis in any of the strains, and concluded that the activity of p60 is tightly regulated. This failure to detect lysis of cells grown in BHI was also seen by Pine *et al.* (1989). In the context of the results presented here, it would be of interest to look for raised amounts or activity of p60 within the medium after cell lysis.

Though instances of the cell lysis described above appeared to be largely attributed to growth phase, cell lysis was also seen during the continuous culture of *L. monocytogenes* and appeared to be due to a particular condition within the chemostat, namely the degree of cell agitation, controlled directly by the speed of the impeller shaft. For reasons unknown, this problem did not manifest itself for the majority of the time spent using the chemostat, even though the conditions described in Section 2.3.0 were maintained throughout the study. Excessive agitation within chemostats, can cause so called 'shear effects' i.e. physical damage to cells. In the case described here however, the impeller speeds used were not excessive (360-380 RPM), and also the effect was not seen for most of the period that the chemostat was used. Nevertheless, reduction in the speed of the impeller not only prevented the lysis here, but also enabled the cells to grow back to their original mass. Therefore, as a preventative measure the speed of the impeller was decreased [See3.2.2] and, in order to maintain the levels of

dissolved oxygen, the air flow rate was correspondingly increased. These steps were sufficient to prevent any further problems of lysis and no further work was performed to measure the effect of shear on *L. monocytogenes.*

4.2 THE EFFECT OF GROWTH TEMPERATURE ON THE FATTY ACID AND POLAR LIPID COMPOSITION OF *L. monocytogenes*

4.2.0 The fatty acid composition of the *L. monocytogenes* cell membrane

The results presented here (Tables 3.3, 3.4) indicate that the most common fatty acids of *L. monocytogenes* grown in defined medium in continuous culture at 30°C are the branched (*anteiso*-) forms of 15:0 and 17:0, with smaller amounts of *iso*-15:0 and *iso*-16:0 (Table 3.3). These observations are in keeping with those reported for *L. monocytogenes* grown in batch culture in complex medium (Raines *et al.*, 1968; Carroll *et al.*, 1968; Tadayon & Carroll, 1971; Feresu and Jones 1988; Ninet *et al.*, 1992; Puttman *et al.*, 1993; Annous *et al.*, 1995). The findings show that use of continuous culture for the study of *L. monocytogenes* at mesophilic growth temperatures, does not have a dramatic effect on the major fatty acid composition. A similar result was noted by Carroll *et al.*, (1968), who compared the fatty acid content of *L. monocytogenes* in batch and continuously cultured cells and found that there was no significant difference in the proportions of fatty acids, whatever the method of growth. However, although the major fatty acids of *L. monocytogenes* at mesophilic growth temperatures are *ai*-15:0 and *ai*-17:0, it is interesting to note that Feresu and Jones (1988) showed significantly differing amounts of the two major fatty acids (*ai*-15:0 (27.9%) *ai*-17:0 (31.1%)) compared to the work presented here. Feresu and Jones, (1988) grew the same strain at 30°C with shaking, using BHI broth, and it may be that the differences in the relative amounts of the major fatty acids were either due to the differences related to batch culture or the medium used. Both of the factors can contribute to differences in bacterial fatty acid and lipid content [See 1.11]. Carroll *et al.*, (1968) did not find any differences in the fatty acid content of

batch and continuously cultured cells of *L. monocytogenes* grown in a complex medium at 37°C, but a later study by Tadayon and Carroll, (1971) noted that the fatty acid content of *L. monocytogenes* differed according to the growth phase of the culture and the nature of the culture medium used.

4.2.1 Growth temperature-induced changes in the fatty acid composition of *L. monocytogenes*

The role of fatty acids in maintaining the integrity of the bacterial membranes in response to a decrease in growth temperature has been considered in detail for a range of psychrophilic and psychrotrophic bacteria, including *L. monocytogenes* in the Introduction of this thesis [See 1.10]. The results of this study show that the most striking effect of a reduction of temperature is a progressive decrease in the levels of *ai*-17:0 at temperatures below 30°C, accompanied by a small rise in *ai*-15:0 and fatty acids ≤15 carbons in length (Table 3.3). These results are in keeping with previous reports (Puttman *et al.*, 1993; Annous *et al.*, 1995). However, because these reports were of studies with *L. monocytogenes* grown on solid complex medium, (Puttman *et al.*, 1993), or in batch culture (Annous *et al.*, 1995), caution had to be exercised in interpreting these changes as being solely attributable to growth temperature. Despite the similarities between the work presented here and the previously published work (Puttman *et al.*, 1993; Annous *et al.*, 1995), the use of continuous culture and a defined medium allows changes in fatty acid composition, to be directly correlated to growth temperature.

The rise in the quantity of fatty acids ≤15 carbons in length, and the reduction in *ai*-17:0 seen at 10°C (Table 3.3) does, however support the suggestion by Annous *et al.*, (1995) that *L. monocytogenes* may able to maintain membrane function at low temperatures by a general reduction in the length of its fatty acids, rather than synthesising unsaturated fatty acids as has been reported for some organisms (e.g. Marr and Ingraham, 1962; Chan, 1971; Wilkins, 1982; Russell, 1990). Additionally, the temperature mediated decrease in the amounts

of *ai*-17:0 and, to a lesser extent, *i*-15:0 has the effect of reducing the ratios of *ai*-17:0 to *ai*-15:0 and *i*-15:0 to *ai*-15:0 with decreasing temperature. This variation in the relative amounts of different membrane fatty acids with respect to temperature, in order to maintain membrane fluidity, has been seen with other bacteria [See 1.10.3].

Not all the fatty acids in this study were identified. A number of the fatty acids, present in relatively low quantities, and grouped in the ≤15 carbons and ≥16 carbons in length categories, were diffecult to identify using the standard mixture. However, as a marked increase in the amount of fatty acids ≤15 carbons in length was seen, an attempt was made to identify the individual fatty acids present [See Table 3.4, Appendix II]. This proved problematic as, though tentative identification in terms of carbon number could be made, only three fatty acids were identified by direct comparison with standards. These were 12:0, 14:0 and 2OH-14:0. Feresu and Jones (1988) indicated that the fatty acids, *i*-13:0, *ai*-13:0, and *i*-14:0, were present in *L. monocytogenes* NCTC 7973 grown at 30°C. It is possible that the fatty acids present in the ≤15 carbon length group tentatively identified in terms of their number of carbons only may well include the fatty acids identified by Feresu and Jones (1988). However the lack of a range of smaller chain unsaturated, *anteiso-* or *iso-* FAMES in the standard mixture employed, prevented the positive identification of these, and other fatty acids (See Figure 3.5).

Other authors have noted the presence small quantities of unsaturated fatty acids, usually 18:1, but also 16:1, in *L. monocytogenes* (See Raines *et al.*, 1968; Feresu and Jones, 1988; Ninet *et al.*, 1992; Russell *et al.*, 1995). Of the two other studies that also considered the effect of temperature on fatty acids of *L. monocytogenes*, no indication of the presence of unsaturated fatty acids was given by Annous *et al.*, (1995) and although the presence of 18:1 and 18:2 fatty acids was noted by Puttman *et al.*, (1993), no changes in the quantity, accompanying a decrease in

the growth temperature were observed. The study by Tadayon and Carroll, (1971) also looked at the effect of growth temperature, and though this work is of little use when considering changes in the amounts of *anteiso-/ iso-* branched chain fatty acids with respect to temperature, because the authors did not differentiate between the type of branching, it is possible to look at the effect of temperature on the amount of 18:1 present. Although the fatty acid only ever seems to be present in small amounts, there did appear to be some increase in the amount produced at lower growth temperatures.

The results presented here indicated that on the basis of comparing FAME retention times, no 18:1, or 16:1 was present at any of the growth temperatures studied, and the major response to temperature of the fatty acids of *L. monocytogenes* is a reduction in *ai*-17:0. It is possible that had a more detailed study, been carried out possibly combining mass spectrometry with gas-liquid chromatography the definitive answer as to the precise chemical composition of the unidentified fatty acids present within *L. monocytogenes* would be known.

4.2.2 The effect of growth temperature on the polar lipid composition of *L. monocytogenes*

The polar lipid content of *L. monocytogenes* at temperatures of 30, 20, and 10°C was assessed using thin layer chromatography (TLC). The use of TLC enabled a qualitative study to be made on the polar moieties present on the polar lipids of *L. monocytogenes*, and in the case of two polar lipids, a comparison with known standards enabled tentative identification to be made. The results of this study, at each of the growth temperatures were found to differ from previously published results for the polar lipids of *L. monocytogenes.* These studies have only been performed on cells grown at mesophilic growth temperatures, and indicated that the polar lipid composition was comprised of phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), galactosyl-glucosyldiacylglycerol, and an uncharacterised phosphoglycolipid (PGL) (Carroll *et al.*, 1968; Kosaric and

Carroll, 1971; Shaw, 1974). A more recent study, (Russell *et al.*, 1995) indicated that the major polar lipid was DPG, with PG, and PGL present in smaller quantities. The results presented here however, differ in a number of ways. When a non-specific ('catch all') polar lipid stain was employed, DPG and PG appeared to be present at all growth temperatures, as were two other polar lipids which, with the aid of specific polar lipid stains were tentatively identified as a phospholipid and a glycolipid. By comparing the relative positions of lipid spots identified using specific stains the possibility of an amino- group present on the glycolipid was also indicated [See 3.3.1]. In addition the use of specific stains only indicated the presence of further polar lipids. These were a glycolipid present at each growth temperature (spot '7' Figures 3.13 (a)-(c)), and a further glycolipid present at 10°C only (spot '8' Fig 3.13(c)). It is possible that differences in the sensitivity of the stains used account for the presence of these spots using the specific stain (α-naphthol), but not when the non-specific stain (dodecamolybdophosphoric acid) was used.

The differences in the polar lipid composition of *L. monocytogenes* in this, compared to other studies, are of interest. It is possible that the unidentified phospholipid seen is bisphosphatidyl-glyceryl phosphate, noted by Kosaric and Carroll, (1971) only. There have been no previous reports of any amino-containing polar lipids in *L. monocytogenes.* Previous reports have indicated the presence of a glycolipid and a phosphoglycolipid in *L. monocytogenes* (Carroll *et al.*, 1968; Kosaric and Carroll, 1971; Shaw, 1971; Russell *et al.*, 1995). This study indicates a glycolipid present at each of the growth temperatures when the specific stain is used (Figure 3.13(a)-(c)) and also a sugar containing polar lipid present using both a non-specific and specific stain (Figure 3.10(a)-(c), Figure 3.13(a)-(c)).

In studies of the polar lipids of *L. monocytogenes* both Carroll *et al.*, (1968) and Kosaric and Carroll, (1971) used cells either grown under continuous culture or

batch conditions in a complex medium at 37°C by Holmstrom (1967). Though Carroll *et al.*, (1968) found no difference in the lipid content of *L. monocytogenes* cells grown by either method, differences related to culture conditions have been seen in other studies, with the effect of growing the cells in batch, rather than continuous culture conditions, appearing to predominantly change relative quantities and proportions of polar lipids, rather than actual polar lipids present (e.g. Oliver and Colwell, 1973; Harwood and Russell, 1984). As all the studies of *L. monocytogenes* polar lipids appear to have used a complex medium, it is possible that the use of a defined medium contributes to differences from the different polar lipids seen here. The type of culture medium have been shown to effect the fatty acids of *L. monocytogenes* (Tadayon and Carroll, 1971), and the polar lipid composition of other organisms (Ratledge and Wilkinson, 1988)

Bhakoo and Herbert, (1979) showed a marked increase in the phospholipid content of psychrophilic *Vibrio,* spp. in response to a reduction in growth temperature. Similarly, Okuyama (1969) showed an increase in phospholipid synthesis at low temperatures in *E. coli*, and also Russell (1974); and Hunter and Rose (1972), noted slight differences in the amounts, but not proportions of polar lipids present in *Micrococcus cryophilus,* and *Saccharomyces cervisiae* when grown at low temperatures. None of these publications indicated the presence of a new polar lipid at low temperatures only. In view of the qualitative nature of the study presented here, and its detection only when a specific stain is used, it is possible that the glycolipid seen at 10°C is only visible because it is synthesised in greater (detectable) quantities in response to a decrease in growth temperature. Therefore the use of alternative methods of analysing polar lipids such as the quantitative method described by Bhakoo and Herbert, (1979), may have provided further evidence as to the presence or absence of the glycolipid at higher growth temperatures, or its synthesis in raised quantities at low growth temperatures.

Further information on temperature mediated alterations to phospholipids could have been gained by studying the *sn*-1/*sn*-2 distribution of fatty acids on the glycerol moiety of a phospholipid, as differences can have a bearing on the liquid-crystalline-gel phase transition temperature (Russell, 1989). Studies have been performed on the distribution of the fatty acids in *L. monocytogenes* at 37°C by Kosaric and Carroll, (1971) who showed that the branched chain 17:0 fatty acids are preferentially esterified to position *sn*-1, whilst branched chain 15:0 are present at position *sn*-2. These workers did not look at the types of branching, and, as they studied the organism at 37°C only did not look at the effect of a reduction in growth temperature (and therefore the effect of a reduction in the amount of *ai*-17:0 present) on the distribution of the fatty acids.

Positional differences of fatty acids on the glycerol backbone of lipids have been shown to significantly effect the phase transition temperature (T_c) of phospholipids (Russell, 1989). The orientation of phospholipids within a membrane is at an slight angle such that the sn-1 'dips' further into the bilayer (Russell, 1989). This differences is exaggerated if the fatty acid present on *sn*-1 is longer than the one on *sn*-2, with the effect that the T_c is higher than for a *sn*-1-short; *sn*-1-long, isomer. Kosaric and Carroll, (1971) showed that at mesophilic temperatures, 17 carbon branched chain fatty acids tend to be present at *sn*-1 in *L. monocytogenes*. Therefore the reduction in *ai*-17:0 seen during a decrease in growth temperature could be an adaptive measure to decrease the T_c of phospholipids present in *L. monocytogenes.*

4.2.3 The role of membrane lipids in maintaining growth of *L. monocytogenes* at low temperature

Changes in membrane phospholipids (particularly the fatty acid acyl chains) are a major response to a reduction in growth temperature in a range of psychrophiles and psychrotrophs (e.g. Herbert, 1989; Russell, 1990, Suutari and Laakso, 1994).

In order to fully discuss the role of changes in membrane fatty acids of *L. monocytogenes* in response to a reduction in the growth temperature seen in this study, consideration must be given to the reason why these changes may occur. The maintenance of a liquid-crystalline state in microbial cell membranes may not only be an adaptation of growth at low temperatures, but also a limiting factor for the minimum growth temperature of mesophilic microorganisms. However, despite the importance of the liquid-crystalline phase for maintenance of cell membrane function, other factors such as the position of fatty acids within a phospholipid [See 4.2.2] and also the type (i.e. straight chain or branched) present may also aid growth at low temperatures.

Studies have indicated that organisms such as *Pseudomonas fluorescens* and *E. coli* alter fatty acids to maintain constant levels of membrane fluidity at differing growth temperatures (Cullen, *et al.*, 1971; Sinensky, 1974). This is not, however, a universal response, organisms such as the psychrotrophic *Pseudomonads* studied by Bhakoo and Herbert, (1980) do not alter their membrane lipid composition in response to a reduction in temperature, and though the fatty acid composition of *Streptococcus faecalis* and *Micrococcus cryophilus* changes with different growth temperatures, membrane fluidity does not remain constant (Wilkins, 1982; Foot *et al.*, 1983).

The major temperature induced response of *L. monocytogenes* fatty acids is a dramatic reduction in the amount of *ai*-17:0 present and an increase in the quantities of small chain (less than 15 carbons in length) (Table 3.3). The presence of these would reduce the overall T_c of the membrane. However even at growth temperature of 10°C, where the quantity of *ai*-17:0 is small, the major fatty acid present *ai*-15:0 has a T_c of 25.8°C (Suutari and Laakso, 1994). Though T_c values of individual fatty acids do not accurately reflect that of the bilayer, it may be other factors may exist that permit adequate functioning of the cell membrane of *L. monocytogenes* at low temperatures. For example, an alteration

within the membrane to reductions in temperature involves a phenomenon known as 'patching' where areas of membrane lipids in the gel phase, force proteins to move laterally within the bilayer (Wunderlich *et al.*, 1975; Furtado *et al.*, 1979). This is thought to be a protective measure and will prevent protein inactivation, its effectiveness is dependant upon the lipid/protein ratio of the membrane of a particular organism. However, in organisms that have high amounts of branched chain fatty acids within the cell membrane, no 'patching' occurs at low growth temperatures, as it appears that such membranes are still able to retain proteins dispersed in the membrane, even if in the gel phase, as the branched chain fatty acids are sufficiently loosely packed, and the membrane less well ordered (Haest *et al.*, 1974; Legendre *et al.*, 1980; Bouvier, 1981). Because *L. monocytogenes* contains predominantly *ai*-15:0 at low growth temperatures it is possible that such a temperature-induced response occurs within the cell membrane, with the maintenance of the bilayer phase, rather than an absolute value of membrane fluidity of paramount importance. Such adjustments, made during thermal transition enabling membrane lipids to be maintained in the same phase, thereby preventing the formation of non-bilayer phases that would impair membrane function are known as 'homeophasic adaptation' (Silvus *et al.*, 1980; Russell, 1989).

4.3 STUDIES ON THE EFFECT OF TEMPERATURE ON CARBOHYDRATE UPTAKE IN *L. monocytogenes*

4.3.0 Uptake and utilisation of different carbohydrates

Previous studies concerning the effect of temperature on the rate of carbohydrate uptake in *L. monocytogenes* have occurred in batch culture, and have looked at a single carbohydrate (glucose) only (Wilkins *et al.*, 1972). It was intended to use continuously cultured cells to look at the effect of growth temperature on glucose uptake [See below]. However, as there have been no studies assessing *L. monocytogenes* uptake of other carbohydrates at different temperatures, a series of experiments were performed to study this. These initial batch culture studies investigated uptake of a range of carbohydrates using the medium of Trivett and Meyer, (1971) at 30 and 10°C. It is possible that the ability of *L. monocytogenes* to exhibit growth at low temperatures may have been enhanced by its ability to utilise a particular carbohydrate at low temperatures only. However, the results of this study indicated that, for the carbohydrates studied, there was no temperature-induced alteration in the ability of the organism to utilise the carbohydrates studied. Though it would have been possible to continue these experiments using different carbohydrates, these were not performed and future uptake experiments concerned the effect of temperature on glucose uptake only.

4.3.1 The use of membrane vesicles for glucose uptake analyses.

Membrane vesicles have not previously been isolated from *L. monocytogenes.* However, studies have made use of membrane vesicles to study uptake of glucose, and other carbohydrates in a range of Gram positive and Gram negative bacteria (Kaback, 1968, 1971, Buckley and Hamilton, 1994). Membrane vesicles are osmotically intact structures, devoid of cytoplasmic constituents but they retain the transport functions of whole cells in the presence of suitable electron donors (e.g. phosphoenol pyruvate (PEP), NADH, NADPH), (Kaback, 1968,1971). The advantage of using vesicles in uptake studies is that individual

uptake systems (e.g. PEP uptake system) can be assessed, and also the absence of any cellular catabolism dispenses with the need for substrate analogues. It was hoped that if membrane vesicles could be isolated from *L. monocytogenes,* they could be used to gauge the effect of temperature on glucose uptake, and also enable studies to be performed on the role of specific uptake pathways. Recent studies have indicated the presence of a PEP uptake pathway for fructose in *L. monocytogenes* (Mitchell *et al.,* 1993). These authors were unable to show PEP uptake for glucose, but this was refuted by Christenson and Hutkins (1994) who provided evidence of a glucose specific PEP uptake system. The use of membrane vesicles to study the PEP uptake system for glucose in *L. monocytogenes* could possibly overcome the uncertainty regarding its presence. PEP uptake systems have been successfully studied using membrane vesicles (Kaback, 1968, 1971).

In addition, Wilkins *et al.,* (1972) showed temperature related differences in substrate uptake in *L. monocytogenes,* if similar differences were seen in glucose uptake rates using membrane vesicles, the presence of further temperature induced uptake systems such as the ATP requiring system noted by Christenson and Hutkins, (1994) could also be looked for.

The results of the experiments presented here, show that membrane vesicles, satisfying the purity criteria of Kaback (1971), could be successfully isolated from *L. monocytogenes* (Figures 3.14, 3.15). However, as it was impossible to accurately quantify the amount of vesicles present [See 3.5], the use of membrane vesicles to measure glucose uptake at different temperatures would be unsatisfactory. Therefore glucose uptake was studied using intact cells of *L. monocytogenes.*

4.3.2 The use of whole cells of *L. monocytogenes* in glucose uptake studies

Whole cells of *L. monocytogenes* have been used in a previous study to look at growth temperature induced changes in glucose uptake (Wilkins *et al.,* 1972). These workers used 2-deoxy-D-glucose, a non-metabolisable analogue of glucose, and cells grown under batch culture conditions and found that for cells harvested in exponential growth phase, cells grown at 10°C took up more 2-deoxy-D-glucose and at a faster rate, than cells grown at 37°C, when uptake was assayed at 10°C and 37°C. The converse was true however, when cells in the stationary growth phase were assayed for 2-deoxy-D-glucose uptake.

The results of the study presented here, used cells grown in a chemostat at 30, 20 and 10°C and the same glucose analogue. A statistically significant increase in the rate and the amount of 2-deoxy-D-glucose uptake was seen with cells grown at 10°C, compared to cells grown at 30°C, when uptake was assayed at 30°C. However there were no significant differences between the same sets of cells when 2-deoxy-D-glucose uptake was assayed at 20°C or 10°C. Although the results presented here only appear to be in partial agreement with those of Wilkins *et al.,* (1972), it is important to consider that these authors also noted that growth temperature mediated 2-deoxy-D-glucose uptake was also significantly affected by the growth phase which, due to continuous culture, was not a consideration here.

These results also indicate that the major factor in uptake of 2-deoxy-D-glucose was assay temperature, with a decrease in assay temperature reducing the amount of 2-deoxy-D-glucose taken up by cells grown at any temperature(Figure 3.15-3.18). A similar finding was seen by Herbert and Bell, (1977) who looked at uptake rates in an obligatory psychrophilic *Vibrio,* SP-1 and concluded that the growth temperature of the organism did not exert any influence in the rate of uptake, though an overall decrease in uptake with increasing growth temperature was seen. In addition, Fukunaga and Russell (1990) compared two Gram negative

psychrotolerant isolates from Antarctica, one with a relatively low optimum growth temperature (psychrophilic) and one with a growth temperature optimum typical of a psychrotroph. Fukunaga and Russell (1990) showed that irrespective of the growth temperature, uptake rates were also greatest at the higher assay temperature (20°C). However, uptake rates of the 'psychrophilic' organism significantly reduced if the organism was grown at 20°C, rather than 5°C, whereas uptake rates were unaffected by growth temperature in the case of the psychrotroph. The results for the organism with a psychrotrophic optimum growth, obtained by Fukunaga and Russell, (1990), are similar to the results seen here for *L. monocytogenes*. It is difficult to draw any conclusions from the cells grown at 20°C. The low values are indicative of an intrinsic fault within the experiment effecting cells at each assay temperature [See 3.6.2]. However cells grown at 20°C also showed that incubation temperature was a factor in the rate and amount of 2-deoxy-D-glucose uptake (Figure 3.18).

Other uptake studies with other psychrotrophs and psychrophiles have showed varying results, Bhakoo and Herbert, (1979, 1980) showed that the rates of glucose uptake in obligately psychrophilic *Vibrio* spp. occurred at a maximal rate at 0°C and decreases with increasing growth temperature, whereas the converse was true for pychrotrophic *Pseudomonas* spp. Russell (1971) showed that uptake in the psychrotroph *Micrococcus cryophilus* occurred at the same rate irrespective of growth temperature, and a similar finding for *Candida* spp. was seen by Baxter and Gibbons, (1962).

4.4 CONCLUSIONS

The results of the studies reported here indicate that:

(1) The defined medium of Trivett and Meyer (1971), not only supported good sequential growth of each of the strains tested, but was also a suitable medium for

the continuous culture of *L. monocytogenes* NCTC 7973 at a range of growth temperatures. In terms of its ease of preparation, it is probably the most suitable defined medium for the culture of *L. monocytogenes* in a chemostat.

(2) Membrane adaptations of *Listeria monocytogenes* NCTC 7973 at low growth temperatures appear to occur chiefly by reducing the quantity of *ai*-17:0 present and also by increasing both the quantity of fatty acids less than 15 carbons in length and the amount of *ai*-15:0 present. These results are comparable with those previously reported for the organism (Puttman *et al.,* 1993; Annous *et al.,* 1995).

(3) A further adaptation in the cell membrane to growth at low temperatures appears to be either the synthesis of a glycolipid at 10°C only, or its presence in raised quantities at low temperatures. In addition the major polar lipids seen in this study (DPG, PG, two glycolipids and an uncharacterised phospholipid) are different from previous reports (Carroll, *et al.,* 1968; Kosaric and Carroll, 1971; Russell *et al.,* 1995).

(4) Temperature mediated differences in 2-deoxy-D-glucose uptake appear to be due to the assay temperature, rather than the cell growth temperature. Evidence of a reduction in the growth temperature enhancing the ability of the organism to take up 2-deoxy-D-glucose which has been reported previously (Wilkins *et al.,* 1972), was confined to cells assayed at 30°C only.

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APPENDIX I

Raw data for GLC of FAMES

Percentage values of individual FAME samples for continuously cultured cells of *L. monocytogenes* NCTC 7973 grown at 30°C.

	Percentage of total fatty acids					
Sample \ FAMES	≤15:0	i-15:0	ai-15:0	i-16:0	ai-17:0	≥16:0
HP13	9.0	1.8	66.1	0.8	20.5	1.1
XHP1-B	1.3	6.2	69.7	1.6	19.6	1.3
XHP7-F	12.38	5.2	60.4	1.7	13.2	6.3
XHP1-I	10.9	3.9	57.9	1.1	17.4	8.5
HP132	1.95	1.8	71.4	1.0	23.5	0.4
XHP12	8.5	1.1	59.1	8.1	14.7	7.8
Average	7.3	3.3	64.1	2.4	18.1	4.2
St. dev[1]	4.6	2.1	5.8	2.8	3.8	3.7
St. error[2]	1.9	0.8	2.4	1.1	1.6	1.5

[1]Standard Deviation. [2]Standard Error

Percentage values of individual FAME samples for continuously cultured cells of *L. monocytogenes* NCTC 7973 grown at 20°C

	Percentage of total fatty acids					
Sample \ FAMES	≤15:0	i-15:0	ai-15:0	i-16:0	ai-17:0	≥16:0
HP17	14.4	2.3	72.0	1.6	9.0	0.9
HP17b	14.3	2.3	72.8	2.7	9.3	0.0
XHP9A	2.8	4.1	80.4	0.0	12.0	0.7
XHP3K	18.3	3.1	65.1	0.0	11.8	1.3
XHP4L	14.4	3.4	65.8	0.0	10.6	5.4
Average	12.8	3.0	71.2	0.9	10.5	1.6
St. dev[1]	5.9	0.8	6.2	1.2	1.4	2.1
St. error[2]	2.4	0.3	2.5	0.5	0.6	0.9

[1]Standard Deviation. [2]Standard Error

Percentage values of individual FAME samples for continuously cultured cells of *L. monocytogenes* NCTC 7973 grown at 15°C

	Percentage of total fatty acids					
Sample \ FAMES	≤15:0	i-15:0	ai-15:0	i-16:0	ai-17:0	≥16:0
HP16	11.0	2.2	75.0	1.8	5.5	4.1
HP16b	6.1	2.4	81.0	1.9	5.9	2.1
XHP16	8.9	1.2	73.3	1.9	6.5	8.5
XHP13	13.0	0.6	57.0	3.1	7.3	5.1
Average	9.7	1.6	71.6	2.2	6.3	4.9
St. dev[1]	3.0	0.8	10.3	0.6	0.8	2.7
St. error[2]	1.2	0.3	4.2	0.3	0.3	1.1

[1]Standard Deviation. [2]Standard Error

Percentage values of individual FAME samples for continuously cultured cells of *L. monocytogenes* NCTC 7973 grown at 10°C

	Percentage of total fatty acids					
Sample.\..FAMES	≤15:0	i-15:0	ai-15:0	i-16:0	ai-17:0	≥16:0
HP15	18.0	0.8	72.9	1.2	4.4	3.2
HP15b	20.9	2.8	64.3	1.1	3.6	6.9
XHP10G	27.8	1.0	64.5	0.0	4.8	2.0
XHP6H	9.7	1.2	71.2	1.1	11.1	5.7
XHP7	30.9	1.2	54.2	6.3	4.4	3.0
XHP18	23.5	1.2	58.7	4.7	3.4	8.5
Average	21.8	1.4	64.3	2.4	5.3	5.1
St. dev[1]	7.5	0.7	7.1	2.5	2.9	4.9
St. error[2]	3.1	0.3	1.0	1.2	1.2	1.0

[1]Standard Deviation. [2]Standard Error

APPENDIX II

Raw data for fatty acids grouped ≤15 carbons in length

Fatty acids less than or equal to 15C in length in FAME samples of cells grown at 30°C.

Sample \ Peak*	a	b	c		d		e	f	g	h	j
HP13	14.85[1]	18.35	19.43	ND	20.23	ND	21.9	24.47	24.94	25.11	28.47
	(0.2)	(2.7)	(2.2)		(1.7)		(0.2)	(0.1)	(0.6)	(1.0)	(0.3)
XHP1-B	14.84[1]	ND	ND	ND	20.26	ND	ND	24.49	ND	ND	ND
	(0.42)				(0.46)			(0.501)			
XHP7-F	14.92[1]	ND	ND	ND	20.28	ND	ND	24.49	ND	ND	ND
	(0.34)				(11.2)			(0.84)			
XHP1-I	14.87[1]	ND	19.52	19.63	20.24	ND	ND	24.47	25.03	25.11	ND
	(0.9)		(1.8)	(2.9)	(1.3)			(0.41)	(0.6)	(2.9)	
HP13-2	14.12[1]	ND	19.49	ND	ND	ND	ND	ND	ND	ND	28.55
	(0.44)		(0.49)								(1.02)
XHP12	14.71[1]	ND	ND	19.96	20.20	22.32	ND	ND	24.78	25.12	ND
	(1.5)			(4.7)	(3.5)	(0.1)			(0.1)	(0.1)	

Values shown are corrected using ai-15:0 as an internal standard. * Refers to the peak labelling system used for Sample HP13 in Figure 3.6 & Table 3.4 [See Section 3.3.0]. ND Peak not detected. Figures in parentheses indicate % of total fatty acid values, other values refer to retention times (min). [1] Fatty acids with a Rt values less than that of the first FAME standard eluted (See Figure 3.5)

Fatty acids less than or equal to 15C in length in FAME samples of cells grown at 10°C.

Sample \ Peak*	a			b	c			d		e	f	g	h	i	
HP15	14.82[1]	ND	ND	18.3	19.51	ND	ND	20.2	ND	21.87	24.29	25.0	25.06	25.41	ND
	(1.6)			(1.3)	(5.1)			(3.4)		(1.5)	(1.4)	(1.5)	(1.9)	(0.3)	
HP15b	14.85[1]	ND	ND	ND	ND	ND	20.07	20.24		21.9	ND	ND	25.09	25.45	26.37
	(0.6)						(5.2)	(4.5)		(1.6)			(3.3)	(0.7)	(5.0)
XHP10G	14.84[1]	ND	ND	ND	ND	19.78	ND	ND	ND	23.7	ND	ND		ND	ND
	(2.8)					(21.2)				(3.8)					
XHP6H	14.81[1]	ND	ND	ND	ND	19.67	ND	20.2	ND	21.86	ND	ND	25.06	ND	ND
	(0.44)					(2.3)		(1.1)		(1.0)			(4.88)		
XHP17	14.69[1]	16.88	17.03	ND	19.47	ND	ND	20.19	ND	21.82	ND	24.87	25.09	ND	ND
	(2.77)	(0.86)	(2.61)		(12.47)			(4.08)		(1.1)		(4.04)	(3.03)		
XHP18	14.68[1]	ND	17.12	ND	19.59	19.6	ND	20.17	ND	21.80	ND	24.86	25.08	ND	ND
	(0.92)		(2.1)		(4.3)	(3.6)		(1.8)		(1.8)		(3.91)	(5.1)		

Values shown are corrected using ai-15:0 as an internal standard. * Refers to the peak labelling system used for Sample HP15 in Figure 3.6 & Table 3.4 [See Section 3.3.0]. ND Peak not detected. Figures in parentheses indicate % of total fatty acid values, other values refer to Retention times (min). [1] Fatty acids with a Rt values less than that of the first FAME standard eluted (See Figure 3.5)

APPENDIX III

Raw data for whole cell 2-deoxy-D-glucose uptake experiments

CELLS GROWN AT 30°C

Cell dry weight 0.2125mg/ Reaction

Assayed at 30°C

Time (hrs)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Mean 1-5	S.Error
0	4.84	5.87	3.88	4.31	-	4.73	0.43
0.5	10.75	10.93	16.92	13.24	-	12.96	1.4
1	12.07	11.44	11.55	13.15	-	12.05	0.39
2	9.25	9.32	12.96	9.21	-	10.18	0.93

Assayed at 20°C

Time (hrs)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Mean 1-5	S. Error
0	4.84	5.87	3.88	4.31	-	4.73	0.43
0.5	11.47	8.64	10.29	11.94	-	10.58	0.74
1	9.5	10.98	9.53	9.81	-	9.96	0.35
2	10.84	9.22	11.27	8.93	-	10.07	0.58

Assayed at 10°C

Time (hrs)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Mean 1-5	S.Error
0	4.84	5.87	3.88	4.31	-	4.73	0.43
0.5	6.59	8.85	6.86	7.38	-	7.42	0.50
1	7.79	8.11	6.6	6.29	-	7.2	0.44
2	10.05	8.22	10.27	9.33	-	9.47	0.46

CELLS GROWN AT 20°C

Cell dry weight 0.2125mg/ Reaction

Assayed at 30°C

Time (hrs)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Mean 1-5	S.Error
0	4.44	3.61	4.11	3.94	4.52	4.12	0.17
0.5	9.45	9.06	6.7	7.86	8.31	8.28	0.48
1	6.10	7.5	9.32	7.72	7.49	7.62	0.51
2	6.76	8.18	8.25	8.35	8.59	8.02	0.32

Assayed at 20°C

Time (hrs)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Mean 1-5	S.Error
0	4.44	3.61	4.11	3.94	4.52	4.12	0.17
0.5	4.33	6.74	5.54	4.82	4.58	5.2	0.43
1	5.00	5.19	4.87	4.79	4.70	4.91	0.09
2	5.49	5.06	6.61	6.47	5.55	5.84	0.3

Assayed at 10°C

Time (hrs)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Mean 1-5	S.Error
0	4.44	3.61	4.11	3.94	4.52	4.12	0.17
0.5	6.52	5.13	3.91	4.88	6.09	5.3	0.46
1	5.55	4.11	4.35	4.85	4.33	4.64	0.258
2	5.24	8.26	4.62	4.84	5.54	5.7	0.66

CELLS GROWN AT 10°C

Cell dry weight 0.2mg / Reaction

Assayed at 30°C

Time (hrs)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Mean 1-5	S.Error
0	5.93	4.31	8.33	5.55	6.59	5.59	0.66
0.5	10.12	12.87	10.68	11.9	-	11.39	0.62
1	15.47	18.61	14.22	14.66	-	15.74	0.99
2	13.46	14.0	13.4	12.47	-	13.34	0.32

Assayed at 20°C

Time (hrs)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Mean 1-5	S.Error
0	5.97	4.31	8.33	4.89	6.59	5.44	0.702
0.5	7.54	15.91	7.85	10.73	8.17	9.98	1.57
1	10.11	19.22	10.88	9.68	9.86	11.45	1.83
2	10.06	25.49	11.06	12.64	11.06	14.06	2.88

Assayed at 10°C

Time (hrs)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Mean 1-5	S.Error
0	5.93	4.31	8.33	4.89	6.59	5.43	0.70
0.5	6.96	7.36	13.35	8.18	5.89	8.35	1.30
1	8.29	9.58	13.35	10.86	7.15	9.85	1.07
2	10.49	8.77	19.8	10.21	7.14	11.28	2.21

Counting Efficiency

2μl of 25μCi/ml gives 83403.5cpm Therefore 0.05μCi added

If 1Ci gives 2.22×10^{12} or 1μCi gives 2.22×10^{6}

Counting efficiency is $\frac{83403 \times 20}{2.22 \times 10^{6}}$ (To get 1μCi)

$=0.751$ (75%)

Calculation of initial uptake rate [See 3.6.0]

Each Reaction has 0.1μCi 'hot' 2-deoxy-D-glucose and 0.5mMol 'cold' 2-deoxy-D-glucose and a mean of 0.2mg cells (dry weight).

1. 2μl of 25μCi/ml gives 83403.05cpm
2. 1ml of 25μCi=41701525 cpm (x500)
3. 0.1μCi/ml=166806.1cpm (Total in Reaction)

4. From reaction, mean of 248 (but only 75% efficiency)
   =330dpm

5. Therefore if x μCi/ml=330dpm from 3 can calculate x.
   $x=1.978 \times 10^{-4}$μCi/ml=330dpm

6. 1.978×10^{-4}μCi/ml=330dpm
   Specific activity of 2-deoxy-D-glucose is 299μCi/μM.
   Therefore amount of 'hot' 2-deoxy-D-glucose taken up in the first 15sec is $1.978 \times 10^{-4}/299 = 6.61 \times 10^{-7}$μM.

7. Start concentration of 'cold' 2-deoxy-D-glucose in reaction is 0.5mMol.
   Therefore assuming that it is taken up at the same rate as 'hot' 2-deoxy-D-glucose,
   To get the total amount of uptake divide 0.5 by the same factor used in 5.

ie (166806.1/330=505.47)

9.891×10^{-4} mM 2-deoxy-D-glucose uptake / 0.2mg drywtcells.

So value uptake value is 4.94×10^{-3} mM 2-deoxy-D-glucose / mg dry wt cells or 4.94 μM 2-deoxy-D-glucose / mg dry wt cells

8. Total amount of 'hot' & 'cold' 2-deoxy-D-glucose taken up is:

$4.94 + (6.61 \times 10^{-7} \times 5) = 4.940003305$μM 2-deoxy-D-glucose /mg cells

SPSS STATISTICAL ANALYSIS OF 2-DEOXY-D-GLUCOSE UPTAKE

Effect of growth temperature on cells grown at 10°C and 30°C

Assayed at 10°C

Effect of:	F value	P(%)	Sig*	Page**
Growth temp	1.37	0.28	-	24
Time	16.45	0.000	+	28
Temp+Time	0.53	0.664	-	28

* Values Significant if P<0.05
** Refers to page number of statistical analysis (Printout 1)

Assayed at 20°C

Effect of:	F value	P(%)	Sig*	Page**
Growth temp	1.04	0.343	-	30
Time	11.61	0.000	+	34
Temp+Time	1.2	0.335	-	34

* Values Significant if P<0.05
** Refers to page number of statistical analysis (Printout 1)

Assayed at 30°C

Effect of:	F value	P(%)	Sig*	Page**
Growth temp	8.58	0.026	+	36
Time	40.10	0.000	+	40
Temp+Time	4.05	0.023	+	40

* Values Significant if P<0.05
** Refers to page number of statistical analysis (Printout 1)

Effect of assay temperature on cells grown at 10°C and 30°C

Effect of:	F value	P(%)	Sig*	Page**
Assay temp[1]	3.76	0.039	+	16
Time	47.51	0.000	+	20
Assay+Time	3.2	0.008	+	20

* Values Significant if P<0.05
** Refers to page number of statistical analysis (Printout 2)
[1]Comparison of 3 Assay temperatures 10, 20 and 30°C

Effect of Assay temperature on growth at 20°C

Effect of:	F value	P(%)	Sig*	Page**
Growth temp	63.64	0.000	+	22
Time	21.98	0.000	+	26
Temp+Time	4.15	0.003	+	26

* Values Significant if P<0.05
** Refers to page number of statistical analysis (Printout 2)

SPSS STATISTICAL ANALYSIS

Printout 1

SPECIAL NOTE

THIS ITEM IS BOUND IN SUCH A MANNER AND WHILE EVERY EFFORT HAS BEEN MADE TO REPRODUCE THE CENTRES, FORCE WOULD RESULT IN DAMAGE

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 22   /design
 23

9 Mar 97 SPSS Release 4.0 for IRIS                                    Page 23
0:01:02  Leicester University          MIPS R3000       IRIX 4.0.1

:  1

* * * * * A N A L Y S I S   O F   V A R I A N C E * * * * * *


        9 cases accepted.
        0 cases rejected because of out-of-range factor values.
        1 case rejected because of missing data.
        2 non-empty cells.

        1 design will be processed.

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9 Mar 97 SPSS Release 4.0 for IRIS                                    Page 24
0:01:02  Leicester University          MIPS R3000       IRIX 4.0.1


* * * * * A N A L Y S I S   O F   V A R I A N C E -- DESIGN   1 * * * * * *

ests of Between-Subjects Effects.

Tests of Significance for T1 using UNIQUE sums of squares
Source of Variation          SS      DF        MS         F  Sig of F

WITHIN CELLS              126.44       7     18.06
G                          24.71       1     24.71      1.37      .280

9 Mar 97 SPSS Release 4.0 for IRIS                                    Page 25
0:01:02  Leicester University          MIPS R3000       IRIX 4.0.1


* * * * * A N A L Y S I S   O F   V A R I A N C E -- DESIGN   1 * * * * * *

ests involving 'TIME' Within-Subject Effect.


Mauchly sphericity test, W =       .19957
Chi-square approx. =              9.22186 with 5 D. F.
Significance =                      .101

Greenhouse-Geisser Epsilon =       .54062
Huynh-Feldt Epsilon =              .78074
Lower-bound Epsilon =              .33333

VERAGED Tests of Significance that follow multivariate tests are equivalent to
nivariate or split-plot or mixed-model approach to repeated measures.
psilons may be used to adjust d.f. for the AVERAGED results.

- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

9 Mar 97 SPSS Release 4.0 for IRIS                                    Page 26
0:01:02  Leicester University          MIPS R3000       IRIX 4.0.1


* * * * * A N A L Y S I S   O F   V A R I A N C E -- DESIGN   1 * * * * * *

EFFECT .. G BY TIME
Multivariate Tests of Significance (S = 1, M = 1/2, N = 1 1/2)
```

Test Name	Value	Exact F	Hypoth. DF	Error DF	Sig. of F
Pillais	.53485	1.91643	3.00	5.00	.245
Hotellings	1.14986	1.91643	3.00	5.00	.245
Wilks	.46515	1.91643	3.00	5.00	.245
Roys	.53485				

Note.. F statistics are exact.

- -

* * * * * A N A L Y S I S O F V A R I A N C E -- DESIGN 1 * * * * * *

EFFECT .. TIME

Multivariate Tests of Significance (S = 1, M = 1/2, N = 1 1/2)

Test Name	Value	Exact F	Hypoth. DF	Error DF	Sig. of F
Pillais	.83451	8.40437	3.00	5.00	.021
Hotellings	5.04262	8.40437	3.00	5.00	.021
Wilks	.16549	8.40437	3.00	5.00	.021
Roys	.83451				

Note.. F statistics are exact.

- -

* * * * * A N A L Y S I S O F V A R I A N C E -- DESIGN 1 * * * * * *

ests involving 'TIME' Within-Subject Effect.

AVERAGED Tests of Significance for MEAS.1 using UNIQUE sums of squares

Source of Variation	SS	DF	MS	F	Sig of F
WITHIN CELLS	48.57	21	2.31		
TIME	114.14	3	38.05	16.45	.000
G BY TIME	3.71	3	1.24	.53	.664

2

* * * * * A N A L Y S I S O F V A R I A N C E -- DESIGN 1 * * * * * *

9 cases accepted.
0 cases rejected because of out-of-range factor values.
1 case rejected because of missing data.
2 non-empty cells.

1 design will be processed.

- -

* * * * * A N A L Y S I S O F V A R I A N C E -- DESIGN 1 * * * * * *

sts of Between-Subjects Effects.

ests of Significance for T1 using UNIQUE sums of squares

ource of Variation	SS	DF	MS	F	Sig of F
ITHIN CELLS	167.65	7	23.95		
	24.81	1	24.81	1.04	.343

* * * * * A N A L Y S I S O F V A R I A N C E -- DESIGN 1 * * * * * *

sts involving 'TIME' Within-Subject Effect.

auchly sphericity test, W = .04656
hi-square approx. = 17.54995 with 5 D. F.
ignificance = .004

reenhouse-Geisser Epsilon = .42478
uynh-Feldt Epsilon = .55126
ower-bound Epsilon = .33333

ERAGED Tests of Significance that follow multivariate tests are equivalent to
ivariate or split-plot or mixed-model approach to repeated measures.
silons may be used to adjust d.f. for the AVERAGED results.

- -

* * * * * A N A L Y S I S O F V A R I A N C E -- DESIGN 1 * * * * * *

FFECT .. G BY TIME
ultivariate Tests of Significance (S = 1, M = 1/2, N = 1 1/2)

est Name	Value	Exact F	Hypoth. DF	Error DF	Sig. of F
illais	.74968	4.99146	3.00	5.00	.058
otellings	2.99488	4.99146	3.00	5.00	.058
ilks	.25032	4.99146	3.00	5.00	.058
oys	.74968				

ote.. F statistics are exact.

- -

* * * * * A N A L Y S I S O F V A R I A N C E -- DESIGN 1 * * * * * *

FFECT .. TIME
ultivariate Tests of Significance (S = 1, M = 1/2, N = 1 1/2)

est Name	Value	Exact F	Hypoth. DF	Error DF	Sig. of F
illais	.84062	8.79043	3.00	5.00	.019
otellings	5.27426	8.79043	3.00	5.00	.019
ilks	.15938	8.79043	3.00	5.00	.019
oys	.84062				

Note.. F statistics are exact.

- -

* * * * * A N A L Y S I S O F V A R I A N C E -- DESIGN 1 * * * * * *

Tests involving 'TIME' Within-Subject Effect.

AVERAGED Tests of Significance for MEAS.1 using UNIQUE sums of squares

Source of Variation	SS	DF	MS	F	Sig of F
WITHIN CELLS	140.54	21	6.69		
TIME	233.11	3	77.70	11.61	.000
G BY TIME	24.05	3	8.02	1.20	.335

3: 3

* * * * * A N A L Y S I S O F V A R I A N C E -- DESIGN 1 * * * * * *

8 cases accepted.
0 cases rejected because of out-of-range factor values.
2 cases rejected because of missing data.
2 non-empty cells.

1 design will be processed.

- -

* * * * * A N A L Y S I S O F V A R I A N C E -- DESIGN 1 * * * * * *

Tests of Between-Subjects Effects.

Tests of Significance for T1 using UNIQUE sums of squares

Source of Variation	SS	DF	MS	F	Sig of F
WITHIN CELLS	15.11	6	2.52		
G	21.61	1	21.61	8.58	.026

* * * * * A N A L Y S I S O F V A R I A N C E -- DESIGN 1 * * * * * *

Tests involving 'TIME' Within-Subject Effect.

Mauchly sphericity test, W = .26329
Chi-square approx. = 6.30185 with 5 D. F.
Significance = .278

Greenhouse-Geisser Epsilon = .66471
Huynh-Feldt Epsilon = 1.00000
Lower-bound Epsilon = .33333

RAGED Tests of Significance that follow multivariate tests are equivalent to
variate or split-plot or mixed-model approach to repeated measures.
lons may be used to adjust d.f. for the AVERAGED results.

- -

* * * * A N A L Y S I S O F V A R I A N C E -- DESIGN 1 * * * * * *

TECT .. G BY TIME
ltivariate Tests of Significance (S = 1, M = 1/2, N = 1)

st Name Value Exact F Hypoth. DF Error DF Sig. of F

llais .89321 11.15252 3.00 4.00 .021
tellings 8.36439 11.15252 3.00 4.00 .021
lks .10679 11.15252 3.00 4.00 .021
rs .89321
te.. F statistics are exact.

- -

* * * * A N A L Y S I S O F V A R I A N C E -- DESIGN 1 * * * * * *

TECT .. TIME
ltivariate Tests of Significance (S = 1, M = 1/2, N = 1)

st Name Value Exact F Hypoth. DF Error DF Sig. of F

llais .95262 26.80529 3.00 4.00 .004
tellings 20.10397 26.80529 3.00 4.00 .004
lks .04738 26.80529 3.00 4.00 .004
rs .95262
te.. F statistics are exact.

- -

* * * * A N A L Y S I S O F V A R I A N C E -- DESIGN 1 * * * * * *

ts involving 'TIME' Within-Subject Effect.

ERAGED Tests of Significance for MEAS.1 using UNIQUE sums of squares

urce of Variation	SS	DF	MS	F	Sig of F
THIN CELLS	50.02	18	2.78		
IE	334.28	3	111.43	40.10	.000
BY TIME	33.77	3	11.26	4.05	.023

5 bytes of memory are needed for MANOVA execution.

```
receding task required .09 seconds CPU time;   .00 seconds elapsed.

24

  23 command lines read.
   0 errors detected.
   0 warnings issued.
   0 seconds CPU time.
   2 seconds elapsed time.
     End of job.
```

SPSS STATISTICAL ANALYSIS

Printout 2

```
ITHIN CELLS          80291.21      36   2230.31
IME                 147037.72       3  49012.57     21.98       .000
 BY TIME             55518.43       6   9253.07      4.15       .003

12 bytes of memory are needed for MANOVA execution.
```



```
eceding task required .10 seconds CPU time;  1.00 seconds elapsed.

13  manova i j k l by h(1,3)
14   /wsfactors=time(4)
15   /design
16
```



```
        1.00

* * * * * A N A L Y S I S   O F   V A R I A N C E * * * * * *


      26 cases accepted.
       0 cases rejected because of out-of-range factor values.
       4 cases rejected because of missing data.
       3 non-empty cells.

       1 design will be processed.
```



```
* * * * * A N A L Y S I S   O F   V A R I A N C E -- DESIGN   1 * * * * * *

sts of Between-Subjects Effects.

ests of Significance for T1 using UNIQUE sums of squares
ource of Variation          SS      DF        MS         F  Sig of F

ITHIN CELLS              380.34      23     16.54
                         124.29       2     62.15      3.76       .039
```



```
* * * * * A N A L Y S I S   O F   V A R I A N C E -- DESIGN   1 * * * * * *

sts involving 'TIME' Within-Subject Effect.


auchly sphericity test, W =       .52917
hi-square approx. =             13.82520 with 5 D. F.
ignificance =                      .017

reenhouse-Geisser Epsilon =       .72348
uynh-Feldt Epsilon =              .87106
ower-bound Epsilon =              .33333

ERAGED Tests of Significance that follow multivariate tests are equivalent to
```

ivariate or split-plot or mixed-model approach to repeated measures.
silons may be used to adjust d.f. for the AVERAGED results.

- -

Mar 97 SPSS Release 4.0 for IRIS Page 18
:08:30 Leicester University MIPS R3000 IRIX 4.0.1

* * * * * A N A L Y S I S O F V A R I A N C E -- DESIGN 1 * * * * * *

FFECT .. H BY TIME
ultivariate Tests of Significance (S = 2, M = 0, N = 9 1/2)

Test Name	Value	Approx. F	Hypoth. DF	Error DF	Sig. of F
Pillais	.68923	3.85605	6.00	44.00	.004
Hotellings	1.94615	6.48716	6.00	40.00	.000
Wilks	.33216	5.14569	6.00	42.00	.000
Roys	.65665				

ote.. F statistic for WILK'S Lambda is exact.

- -

Mar 97 SPSS Release 4.0 for IRIS Page 19
:08:30 Leicester University MIPS R3000 IRIX 4.0.1

* * * * * A N A L Y S I S O F V A R I A N C E -- DESIGN 1 * * * * * *

FFECT .. TIME
ultivariate Tests of Significance (S = 1, M = 1/2, N = 9 1/2)

Test Name	Value	Exact F	Hypoth. DF	Error DF	Sig. of F
Pillais	.83547	35.54557	3.00	21.00	.000
Hotellings	5.07794	35.54557	3.00	21.00	.000
Wilks	.16453	35.54557	3.00	21.00	.000
Roys	.83547				

ote.. F statistics are exact.

- -

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:08:30 Leicester University MIPS R3000 IRIX 4.0.1

* * * * * A N A L Y S I S O F V A R I A N C E -- DESIGN 1 * * * * * *

sts involving 'TIME' Within-Subject Effect.

VERAGED Tests of Significance for MEAS.1 using UNIQUE sums of squares

Source of Variation	SS	DF	MS	F	Sig of F
WITHIN CELLS	300.65	69	4.36		
TIME	620.98	3	206.99	47.51	.000
BY TIME	83.74	6	13.96	3.20	.008

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2.00

* * * * * A N A L Y S I S O F V A R I A N C E -- DESIGN 1 * * * * * *

15 cases accepted.
0 cases rejected because of out-of-range factor values.
0 cases rejected because of missing data.
3 non-empty cells.

1 design will be processed.

- -

* * * * * A N A L Y S I S O F V A R I A N C E -- DESIGN 1 * * * * * *

ests of Between-Subjects Effects.

Tests of Significance for T1 using UNIQUE sums of squares

Source of Variation	SS	DF	MS	F	Sig of F
WITHIN CELLS	5.20	12	.43		
H	55.14	2	27.57	63.64	.000

* * * * * A N A L Y S I S O F V A R I A N C E -- DESIGN 1 * * * * * *

ests involving 'TIME' Within-Subject Effect.

Mauchly sphericity test, W = .60271
Chi-square approx. = 5.42890 with 5 D. F.
Significance = .366

Greenhouse-Geisser Epsilon = .79695
Huynh-Feldt Epsilon = 1.00000
Lower-bound Epsilon = .33333

VERAGED Tests of Significance that follow multivariate tests are equivalent to
nivariate or split-plot or mixed-model approach to repeated measures.
psilons may be used to adjust d.f. for the AVERAGED results.

- -

* * * * * A N A L Y S I S O F V A R I A N C E -- DESIGN 1 * * * * * *

EFFECT .. H BY TIME
Multivariate Tests of Significance (S = 2, M = 0, N = 4)

Test Name	Value	Approx. F	Hypoth. DF	Error DF	Sig. of F
Pillais	.85901	2.76050	6.00	22.00	.037
Hotellings	5.45119	8.17679	6.00	18.00	.000
Wilks	.15313	5.18492	6.00	20.00	.002
Roys	.84464				

Note.. F statistic for WILK'S Lambda is exact.

- -

```
:08:30  Leicester University           MIPS R3000       IRIX 4.0.1

* * * * * A N A L Y S I S   O F   V A R I A N C E -- DESIGN   1 * * * * * *

FFECT .. TIME
ultivariate Tests of Significance (S = 1, M = 1/2, N = 4 )

est Name          Value    Exact F Hypoth. DF   Error DF  Sig. of F

illais           .91867   37.65301       3.00      10.00       .000
otellings      11.29590   37.65301       3.00      10.00       .000
ilks             .08133   37.65301       3.00      10.00       .000
oys              .91867
ote.. F statistics are exact.

 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

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* * * * * A N A L Y S I S   O F   V A R I A N C E -- DESIGN   1 * * * * * *

sts involving 'TIME' Within-Subject Effect.

VERAGED Tests of Significance for MEAS.1 using UNIQUE sums of squares
ource of Variation          SS      DF        MS         F  Sig of F

ITHIN CELLS              28.40      36       .79
IME                      52.00       3     17.33     21.98       .000
 BY TIME                 19.64       6      3.27      4.15       .003

12 bytes of memory are needed for MANOVA execution.

 Mar 97 SPSS Release 4.0 for IRIS                                    Page 27
:08:30  Leicester University           MIPS R3000       IRIX 4.0.1


eceding task required .05 seconds CPU time;  .00 seconds elapsed.

17

  16 command lines read.
   0 errors detected.
   0 warnings issued.
   0 seconds CPU time.
   2 seconds elapsed time.
     End of job.
```