Listeria monocytogenes

detection and behaviour in food and in the environment

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Stellingen

1 Zolang ophopingen niet resulteren in een monocultuur van het gezochte micro-organisme moeten speciale eisen gesteld worden aan (s)electieve isolatiemedia.
Dit proefschrift

2 Cox's wet van verwarring: Het aantal methoden om listeria's te bepalen is M = N + 1, waarin M = het aantal methoden, N = het aantal microbiologen en 1 = de methode in het boek.
LJ Cox, persoonlijke mededeling

3 De aanduiding 'hamburger disease' voor infecties veroorzaakt door *Escherichia coli* O157:H7 wijst te eenzijdig op rundvlees als bron van deze pathogeen.

4 De noodzaak van een ophopings- en bevestigingsstap maakt veel commerciële microbiologische detectiemethoden tot niet meer dan een snelle en dure tussenstap in een traditionele microbiologische bepaling.

5 Bij veel voorverpakte voedingsmiddelen wordt krampachtig geprobeerd door misbruik van het begrip 'vers' iets te claimen wat eigenlijk is voorbehouden aan de ambachtelijke sector.

6 De praktische bruikbaarheid van (extracten van) specerijen of kruiden en bacteriocinen als conserveermiddel is twijfelachtig.

7 Bacteriën delen om te vermenigvuldigen; mensen moeten delen omdát ze zich vermenigvuldigen.

8 Zolang besmetting van gesneden vleeswaren met listeria's niet kan worden uitgesloten, moet de houdbaarheidstermijn van deze produkten worden verkort.
Dit proefschrift

9 Er is dringend behoefte aan een leerstoel levensmiddelenvirologie.

10 Er sterven meer mensen aan liefdesverdriet dan aan listeriose; bovendien is eerstgenoemde kwaal moeilijker te voorkomen.
Naar Uncle Benn in 'More die of heartbreak', Saul Bellow, 1987

11 Persoonlijke en huishoudelijke hygiëne kan bijdragen aan preventie van listeriose.
Dit proefschrift

12 Handen wassen is reguliere geneeskunde.
Céline's proefschrift over Semmelweiss

13 Het is een illusie te denken dat rauwe voedingsmiddelen vrij kunnen zijn van pathogene micro-organismen. Het is daarom noodzakelijk, naast (hygiënische) maatregelen bij oogst, slacht, distributie en verdere verwerking, de consument van jongsaf aan te scholen in het hygiënisch bereiden van voedsel. Het vak 'Verzorging' in het voortgezet onderwijs biedt hiervoor een goede gelegenheid.

14 Het aantal personen dat betrokken is bij het onderzoek naar listeriose is aanzienlijk groter dan het aantal slachtoffers van deze ziekte.

15 Swetsitis bij levensmiddelenmicrobiologen die zich met risicoanalyse bezighouden kan bestreden worden met de door Hollander beschreven therapie voor deze humanose: verandering van milieu, in dit geval van bureau naar praktijk.
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voor mijn moeder

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ABSTRACT

In this thesis, *Listeria monocytogenes*, a bacterial pathogen was studied, with emphasis on the detection and behaviour in food and environment.

Epidemics of foodborne listeriosis have raised concern about the incidence of *L. monocytogenes* in foods. In the past 10-15 years listeriosis has emerged as a foodborne illness in a series of large outbreaks from contaminated milk, coleslaw, soft cheese and pâté. The organism is ubiquitous in the environment and has been isolated from a variety of raw and ready-to-eat food products.

As there is a lack of reliable enrichment procedures, factors influencing the isolation, confirmation and identification of *L. monocytogenes* were investigated. It was shown that the other (faster growing) listerias can mask the presence of this pathogen in enrichment media. The use of lithium chloride did not overcome this effect. It was also observed that the use of acriflavine in enrichment media affected the isolation of *L. monocytogenes* both directly and indirectly. Because these effects will lead to inferior detection of *L. monocytogenes*, it is worthwhile to introduce an isolation medium on which the pathogen can be differentiated from non-pathogenic listerias. On enhanced hemolysis agar (EHA), *L. monocytogenes* can be distinguished from other listerias on the basis of hemolysis.

The traditional methods for the detection of *L. monocytogenes* are both time consuming and labour intensive. Therefore, rapid test kits for *Listeria* and *L. monocytogenes* have been developed. Differences among the various test kits may be contributed to the enrichment protocols, the detection limits of the tests and the concentration of *Listeria* cells in the samples.

In the second part of this thesis, the behaviour of *L. monocytogenes* in food and environment was investigated.

In the literature contradictory results on the growth of the organism on meat surfaces have been reported. In this study it was shown that growth on raw meat was strain dependent and mainly determined by initial pH and storage temperature. On cooked meat products the growth was not or only slightly affected by the storage atmosphere and the presence of lactic acid bacteria. At the end of shelf life, levels of 10^7 cfu per g could be reached.

Under osmotic stress conditions exogenously added proline, betaine and carntine significantly stimulate growth in laboratory media. Because these compounds are also present in foods, they can contribute to growth of *L. monocytogenes* in various foods at high osmolarities.

In sporadic cases of listeriosis it is often difficult to link food products with this disease, unknown sources may also be responsible for illness. From an investigation in the domestic environment it became clear that *L. monocytogenes* may be present in high numbers.

To be able to control the problems caused by *L. monocytogenes*, international microbiological criteria, based on $< 10^2$ L. monocytogenes cells per gram on sell-by-date should be established. Persons at risk (immuno-compromized, pregnant) should be strongly advised not to eat food products likely to contain the pathogen. Meanwhile, more attention should be given to challenge studies, factory ecology, cleaning and disinfection and both personal and domestic hygiene.

CONTENTS

1

GENERAL INTRODUCTION

RR Beumer

Food microbiologists usually consider *Listeria monocytogenes* to be a newly emerged (foodborne) pathogen, but is this really true? In the second edition of Dr. H.P.R. Seeliger's (1961) monograph 'Listeriosis' the foreword was written by Dr.Dr.h.c. K.F. Meyer (Hooper Foundation for Medical Research, San Francisco, California). The text of the first paragraph is quoted below.

"About 44 years ago, shortly after the causative agent of listeriosis had been encountered in wild rodents, there was an epizootic among veldt rodents in the De Aar area along the Tiger River in South Africa. Sand rats were dying in numbers, and an organism was isolated, not virulent for most rodents but fatal to gerbils by ingestion. On the principle prevailing at that time -of casting out devils by Beelzebub- the so-called Tiger River virus, or *Listeria monocytogenes* of today, was used in an effort to destroy gerbils over a belt of South African country some 20 miles long, with some success. Among those entrusted with the control of plaque there were advocates of introducing *Listeria* into other continents for rodent control. This is understandable, because the first proved human infection was not reported until 1929, and the livestock infections, much later still. But there can be hardly any doubt that infection with this organism was associated with illness and death in man and other vertebrates long before the cause was isolated and identified".

1

TAXONOMY

The first description of the causative organism of the syndrome that now is called listeriosis was published in 1926 by Murray, Webb and Swann (1926). The micro-organism was isolated from infected rabbits in the stables of the laboratories in Cambridge. Since a typical monocytogenes (increased quantities of monocytes, a type of white blood cells) was observed in infected animals this organism was named *Bacterium monocytogenes*. One year later the same organism was described by Pirie (1927): he isolated it from a gerbil in South Africa. To honour Lord Lister, and due to the typical liver infections this microorganism could induce in experimentally infected animals, the pathogen was named: *Listerella hepatolyticus*. Being identical, both species were combined to: *Listerella monocytogenes* but in 1940 this species name was changed into *Listeria monocytogenes* (Pirie 1940), since the generic name *Listerella* was already in use. However, some objections were made to the new name: *Listera* was used for an orchid and *Listeria* for one of the diptera. Because the name *L. monocytogenes* was used generally by the bacteriologists of that time, the International Commission of Bacteriological Nomenclature decided to accept this name. After the inclusion of this name in the standard text books of bacteriology, this bacterium was redescribed several times as a new organism (Seeliger 1961).

As Gram-positive, non-sporeforming, catalase positive rods, the genus *Listeria*, having one species: *L. monocytogenes*, was placed in the family *Corynebacteriaceae*. Between 1961 and 1971 three additional species, *L. denitrificans*, *L. grayi* and *L. murrayi* were described (Seeliger and Welshimer 1974). The classification of *L. denitrificans* in this genus was questionable and later this species was transferred to a new genus: *Jonesia* (Jones 1992). *L. grayi* and *L. murrayi* were not sufficiently different from each other to merit seperate species status. A proposal to transfer these species to a new genus: *Murraya* (Stuart and Welshimer 1974) was rejected. Based on the work of Rocourt et al. (1992) both species were combined to *L. grayi*.

From numerous studies it became clear that listerias showed more similarity with lactic acid bacteria than with corynebacteriae, reason to move the genus to Section 14 in Bergey's Manual of Systematic Bacteriology: regular, non-sporing Gram-positive rods (Seeliger and Jones 1986).

In the description of *L. monocytogenes* special attention was given to non-hemolytic strains, non-pathogenic for mice. For these strains Seeliger (1981) proposed a new species: *L. innocua*. Later, DNA-DNA hybridization studies on numerous strains of *L.*

monocytogenes showed the presence of five genomic groups now represented by the species: *L. monocytogenes*, *L. innocua*, *L. ivanovii* (strains first described by Ivanov 1962), *L. seeligeri* and *L. welshimeri* (Rocourt et al. 1982). These results have been reinforced by 16S rRNA sequencing studies of Collins et al. (1991). Multilocus enzyme studies resulted in the detection of two *L. ivanovii* subspecies *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis* (Boerlin et al. 1992). On the basis of current data the genus contains six species representing two closely related, but distinct lines of descent: 1) *L. monocytogenes*, *L. innocua*, *L. ivanovii* subsp. *L. ivanovii*, *L. ivanovii* subsp. *londoniensis*, *L. seeligeri* and *L. welshimeri* and 2) *L. grayi*. The genus is most closely related to the genus *Brochothrix* (Jones 1992).

OCCURRENCE AND DISTRIBUTION IN THE ENVIRONMENT

L. monocytogenes is widespread in the environment and as a consequence of this, raw food products harbour this pathogen in low numbers, and processed foods may be contaminated from the production environment. Because this pathogen is able to growth at refrigeration temperature, a considerable increase in numbers is possible in contaminated products with a long shelf-life at low temperature such as pâté and soft cheeses.

Air

There have been a few reported incidents of listeriosis associated with aerosol infection in man or animals: the inhalation of 'infected dust' (Seeliger 1961) and a case of *Listeria* sepsis following an aspiration pneumonia after eating contaminated coleslaw (Schlech 1984). But no reliable data are available about presence of *Listeria* spp. in air. Air in close proximity of water purification plants may be contaminated with listerias since presence of pathogen indicators was demonstrated during land application of sewage sludge (Pillai et al. 1996).

Water

Listeria has been detected, using MPN techniques, in fresh surface water (2 per 100 ml) and in samples of untreated and treated sewage (up to 1320 per 100 ml). The most frequently isolated species were *L. innocua* and *L. monocytogenes* in sewage, and *L. seeligeri* in samples from ponds and lakes (Watkins and Sleath 1981, Geuenich et al. 1985, Colburn et al. 1990, Bernagozzi et al. 1994). Other species were detected, but to a lesser extent. It has been suggested that waters not receiving sewage effluents have a

low isolation rate of *Listeria* spp. Although not statistically significant, this is supported by coliform and *Escherichia coli* counts being higher in waters that were positive for *Listeria* spp. compared to those which were negative (Dijkstra 1982, Frances et al. 1991, Bernagozzi et al. 1994). Because the numbers of *Listeria* spp. found in sewage are usually low, and enrichment procedures are employed for their detection, it is not possible to confirm a seasonal variation or a decrease in numbers by sewage treatment (MacGowan et al. 1994).

Silage

During the 1960s there was no certainty about a natural reservoir of *L. monocytogenes*. There was some evidence that silage-feeding was connected with outbreaks of listeriosis. Moreover, there was reason to suspect its latent existence among rodents, and in the tick species *Ixodes ricinus*, associated with cows and rodent species (Seeliger 1961). Only a few years later it was shown that the use of silage of inferior quality was of importance in the explanation of clinical outbreaks in animals (Dijkstra 1965).

Listeria spp., including *L. monocytogenes*, were isolated regularly from soil and plants. The percentage of positive samples ranging from 9%-75% (Welshimer 1968, Weis and Seeliger 1975) and especially in decaying (vegetable) material presence of *L. monocytogenes* was demonstrated, which is in agreement with the high numbers found in poor quality silage (Kalač and Woolford 1982). Moreover, it was shown that *Listeria* could survive in soil for up to 295 days (Welshimer 1960).

Faeces

Due to its ubiquity, *Listeria* spp. can also be isolated from faecal samples. The incidence in cattle faeces ranges from a few percent up to 29% of the samples investigated (Kampelmacher and Van Noorle-Jansen 1969 1980, Dijkstra 1965, Ralovich 1984). Higher percentages (up to 51%) were reported by Skovgaard and Morgen (1988). Presence of *Listeria* was also demonstrated in faecal samples (12.3%) from horses (Weber et al. 1994) and other domestic animals (Weber et al. 1995).

Fenlon (1985) reported that birds feeding on sewage had a higher carriage rate of *Listeria* spp. than birds feeding elsewhere. This is supported by the results of a study on the intestinal carriage of pathogens in pigeons in the city of Barcelona (Spain): *Listeria* was only isolated from one of the 400 faecal samples investigated (Casanovas et al. 1995). Listerias were even isolated from the faeces of a high percentage of apparently

healthy adults, diseased patients and their household contacts (Bojsen-Møller 1964, Kampelmacher and Van Noorle-Jansen 1969 1972, MacGowan et al. 1991, Schuchat et al. 1991). In most cases, faecal shedding seems to be caused by oral ingestion of contaminated food rather than by contact with infected animals (Kampelmacher and Van Noorle-Jansen 1969, Schlech 1991, Schuchat et al. 1991). A low incidence of faecal carriage of *Listeria* spp. (1%) was described by MacGowan et al. (1994) probably due to the different isolation methods used. A previous study indicated seasonal distribution in faecal isolation (MacGowan et al. 1991), which was confirmed in this later study, where the predominant species identified were *L. monocytogenes* and *L. innocua*. These results agree with findings of Lamont and Postlethwaite (1986).

DETECTION METHODS

Since it became obvious that *L. monocytogenes* was pathogenic in man and animals, there was a need for reliable detection methods. The first isolation methods were generally based on the direct culture of samples on simple agar media. In case of low numbers of viable *Listeria* cells, isolation was difficult and inoculation into test animals (intraperitoneally or subcutaneously), including embryonated eggs, was recommended. Both promising and discouraging results were described. From experiments with guinea-pigs and mice, Larsen (1969) concluded that the biological method, using test animals, could be of value for detecting small numbers of *Listeria* in samples without competitive microorganisms. However, in presence of contaminants, test animals died of septicaemia, apparently caused by a Gram-negative mixed flora.

Better results were obtained with the cold enrichment procedure. Testing of the same material using the refrigeration technique resulted in more positive samples compared with incubation at elevated temperatures. One of the first reports about the survival of *Listeria* (in brain tissue) at low temperatures was published by Biester and Schwarte (1939). Almost ten years later Gray et al. (1948) described a new technique based on cold enrichment for several weeks at 4°C. Since that time the method has been applied successfully (Gray 1957 1962, Girard and Sbarra 1963). In short the procedure was based on homogenization of the sample in a mortar, to which broth or water was added. From this suspension material was streaked, directly and at regular intervals (days or weeks), on colourless agar media: tryptose- or McBride agar. The incubated plates were examined for the presence of characteristic blue colonies using oblique trans-illumination (Henry 1933). The major disadvantage of this method was the long incubation period

Table 1 A selection of selective agents used in media for the detection of *Listeria* spp.

Selective agents	Reference	Year
Acridine dyes	Bockemühl et al.	1971
Acriflavine	Skalka and Smola	1983
Alkaline pH	Seeliger	1955
Amphotericin B	Simon	1956
Brilliant Green	Robin and Magard	1960
Chloramphenicol	Kampelmacher and Van Noorle-Jansen	1961
Chromium trioxide	Mavrothalassitis	1977
Colistin	Fallon	1985
Cycloheximide	Bearns and Girard	1959
Gallocyanin	Mavrothalassitis	1977
Glycerine-saline	Olson et al.	1953
Guanofuracin	Shimizu et al.	1954
Indigo	Durst and Berencsi	1975
Lithiumchloride	Bearns and Girard	1959
Methylene blue	Despierres	1971
Moxalactam	Lee and McClain	1986
Nalidixic acid	Beerens and Tahon-Castel	1966
Nitrofurazone	McBride and Girard	1960
Nystatin	Braveny and Grote	1973
Polymyxine B	Bojsen-Møller	1963
Phenylethanol	Bearns and Girard	1959
Potassium dichromate	Mavrothalassitis	1977
Potassium tellurite	Gray et al.	1950
Potassium thiocyanate	Lehnert	1964
Propolis	Gronstol and Aspoy	1977
Pyronine	Mavrothalassitis	1977
Sodium chloride	Stenberg and Hammainen	1955
Tartrazine	Durst and Berencsi	1975
Thallium acetate	Kramer and Jones	1969
Thionine	Mavrothalassitis	1977
Trypaflavin	Ralovich et al.	1971
Viomycin chloride	Sagdullaeva	1986

(up to several months). Newer techniques were based on the use of a variety of selective and elective agents in isolation and enrichment media. Results were sooner available compared with the cold enrichment and, for that reason more suitable for routine work. Nevertheless, it was commonly accepted that the sensitivity of the cold enrichment combined with the Henry illumination could not be improved by using other techniques (Girard and Sbarra 1963). None of the methods applied at that time gained universal acceptance and, although numerous selective agents have been proposed for the use in enrichment and/or isolation media since then (see for a selection of these Table 1), up till now there is not a generally accepted method for the detection of *Listeria*.

Direct after the major listeriosis outbreaks in the United States two federal agencies: the United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS), which is responsible for the regulation of meat, poultry and egg products, and the Food and Drug Administration (FDA) of the Department of Health and Human Services (DHHS), which is responsible for other foods, including seafood, both recommended their own methods for the detection of *Listeria* spp. The FDA method, was based on 48h enrichment in *Listeria* Enrichment Broth (LEB), a medium with a low buffering capacity (Lovett et al. 1987), and the USDA method was originally based on a broth formulated by Dominquez Rodriquez et al. (1984) and adapted by Donnelly and Baigent (1986), a medium with sufficient buffering capacity. The USDA method was soon changed from a one stage to a two-stage enrichment, which resulted in a more effective method for the recovery of *L. monocytogenes* from meat samples (McClain and Lee 1988). Due to the qualifications of the agencies it was generally accepted that the USDA method was suitable for meat products and the FDA method for other food products such as dairy products, seafood and vegetables. However, this was never demonstrated: very few studies were done that compared both methods for the same set of samples. Recently, the International Organization for Standardization (ISO), published a draft method for the detection of *L. monocytogenes*, based on the USDA method (Anonymous 1995).

Many solid media have been developed to isolate *Listeria* from enrichment media, or for direct plating methods. One of the first selective media described was McBride agar (MA, McBride and Girard 1960), a medium based on glycine anhydride, lithium chloride and phenylethanol. Blood was added to detect hemolytic strains of *Listeria*. The medium was improved (modified McBride agar, MMA) by adding cycloheximide to inhibit fungi (Lovett et al. 1987) or by adding potassium tellurite, bacitracin, nalidixic

acid and moxalactam (ARS-MMA, Buchanan et al. 1987). At the same time another medium was developed, lithium chloride phenylethanol moxalactam agar (LPM). In comparison with MMA, concentrations of lithium chloride (10x) and moxalactam (4x) were increased (Lee and McClain 1986). Because LPM was more selective than MMA, the use of MMA decreased. The introduction of media containing aesculin and ferric citrate as elective system was a further improvement (Curtis et al. 1989, Van Netten et al. 1988). Comparative studies with a lot of different plating media have been published from that time (Brackett and Beuchat 1989, Golden et al. 1990, Slade 1992, Hayes et al. 1992, Paranjpye et al. 1992). None of these media is generally accepted and all suffer from the disadvantage that *L. monocytogenes* cannot be differentiated from other *Listeria* spp. Probably due to the strict regulations, the media recommended in the FDA (LPM and Oxford agar) and USDA (modified Oxford agar, McClain and Lee 1989) procedures are the most frequently used in the United States. In Europe, PALCAM (Van Netten et al. 1988) and Oxford agar (Curtis et al. 1989) are prefered to LPM. In a comparative study using these two plating media it was concluded that PALCAM agar was consistently more effective in suppressing competitive microorganisms, thus enhancing the possibility of detecting *Listeria* spp. present in low numbers (Gunasinghe et al. 1994).

Recently it has been shown that the incidence of *L. monocytogenes* may be underreported, because presence of this pathogen in enrichment media can be masked by faster growth of other *Listeria* spp. (Petran and Swanson 1993, McDonald and Sutherland 1994). In procedures where all species of a genus are considered to be pathogenic, i.e. *Salmonella*, this will only result in epidemiologically false information and not in missing contaminated samples. In case of detecting *L. monocytogenes*, the only species of this genus pathogenic to humans, the use of isolation media that allow identification of this pathogen in presence of high numbers of other listerias, should be recommended.

AIM AND OUTLINE OF THIS THESIS

The aims of this study are to improve the detection of *L. monocytogenes*, to clarify its behaviour in raw meat and meat products, to investigate the growth possibilities in presence of high salt concentrations and to determine its occurrence in domestic environments. In part A of this thesis (Chapters 1-6), results concerning isolation, confirmation and identification of this pathogen are reported. The behaviour of *L. monocytogenes* in raw and processed meat products, the effect of osmoprotectants on its growth, and the occurrence in domestic environments are described in Part B.

As there is a lack of reliable enrichment procedures for *L. monocytogenes*, attempts have been made to improve the detection of this pathogen. In Chapter 2 the performance of an enrichment medium based on lithium chloride is described. It does not inhibit listerias significantly if lithium chloride is used at concentrations up to 2%, but further comparative studies are required to recommend its use in the present protocols. In Chapter 3 it is shown that the presence of *L. monocytogenes* in the traditional enrichment procedures is masked by other, faster growing, *Listeria* spp. To overcome this problem an isolation medium on which *L. monocytogenes* can be differentiated from other listerias, has been improved and its performance is described in Chapter 4. So-called 'rapid methods' for the detection, confirmation and identification of *Listeria* and/or *L. monocytogenes* are evaluated in Chapter 5 and Chapter 6.

Chapter 7 is a review about the presence and the behaviour of *L. monocytogenes* in food products. Because contradictory results on the growth of the organism on meat surfaces have been published, the factors that influence the growth of this pathogen on raw meat are investigated in Chapter 8. In products with a long shelf life, listerias (present by post process contamination) can grow to unacceptably high levels. In Chapter 9 this is shown for the growth of *L. monocytogenes* on cooked meat products. The effect of the competitive microflora (lactic acid bacteria) is also described in this chapter.

Osmoprotectants such as betaine, a trimethyl amino acid, and proline are involved in the adaptation to high extracellular solute concentrations. Another trimethyl amino acid is carnitine, an important compound in mammalian tissues, involved in lipid metabolism. Chapter 10 describes the effect of proline, betaine and carnitine on the growth of *L. monocytogenes* and *L. innocua* in a minimal medium and in a rich medium with increasing salt concentrations.

Especially in sporadic cases of listeriosis it is difficult to link food products with this disease. It may be possible that other sources of *L. monocytogenes* might be responsible for human listeriosis. The occurrence of this pathogen in domestic environments is reported in Chapter 11. The general discussion, including recommendations for standards and detection of this pathogen, is presented in Chapter 12.

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2

EFFECT OF LITHIUM CHLORIDE AND OTHER INHIBITORS ON THE GROWTH OF *LISTERIA* SPP.

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SUMMARY

The effect of lithium chloride (LiCl) on the growth of *Listeria* was studied in enrichment broths to determine the optimum levels of this inhibitor. *Listeria monocytogenes* could grow at concentrations up to 2%, but above this level, inhibition was noted. *Enterococcus faecalis*, a major competitive organism, was inhibited by low levels of LiCl. Competitive flora capable of growth in the presence of LiCl is normally inhibited by nalidixic acid except for staphylococci. Substrates such as skim milk powder do not affect the inhibition of competitors by LiCl significantly. Acriflavine was shown not to significantly inhibit the *L. monocytogenes* strain studied in the presence of LiCl. The combination of LiCl, acriflavine and nalidixic acid should be studied further in enrichment procedures for *L. monocytogenes*.

2

INTRODUCTION

Lithium chloride has been used traditionally in media for the isolation of *Staphylococcus aureus* from foods (Minor and Marth 1976). The first post-war reference to the use of LiCl was that of Ludlam (1949) for the inhibition of *St. albus* in selective media for *St. aureus*. Zebovitz et al. (1955) improved Ludlam's medium by the inclusion of glycine and potassium tellurite. The media of Vogel and Johnson (1960) (V-J) and of Baird-Parker (1962) employed LiCl at the level of 0.5% recommended by Ludlam (1949) and the tellurite-polymixin-egg yolk medium of Crisley et al. (1964) contained a level of 0.22%.

McBride and Girard (1960) were the first to employ LiCl in a (solid) medium for the isolation of *Listeria monocytogenes* in combination with phenylethanol and glycine, and blood to indicate hemolysin production. Most solid media for isolation of listeria's are modifications of V-J (Buchanan et al. 1989), or McBride *Listeria* agar. Beerens and Tahon-Castel (1966) were the first to incorporate the quinolone antibiotic nalidixic acid into solid media. Later, solid media have been formulated that contain much higher concentrations of LiCl (Curtis et al. 1989, Van Netten et al. 1988) and newer antibiotics such as ceftazidime (Bannerman and Bille 1988, Lee and McClain 1988, Curtis et al. 1989).

Liquid media for the enrichment of listerias have developed along a historical course somewhat different to that of solid media, and have been mostly based on the trypaflavine-nalidixic acid medium of Ralovich et al. (1971). Reviews of the various liquid and solid media and selective agents and methods used for isolation of listerias have been made by Prentice and Neaves (1988) and Cassiday and Brackett (1989).

Despite the fact that LiCl has been used for many years in microbiological media, its mechanism of action on bacterial cells is still poorly understood. Table 1 gives a brief overview of literature concerning the positive and negative effects of lithium ions on microorganisms.

However, few data occur in literature concerning the limiting concentrations of LiCl or other inhibitory compounds for growth of listerias. The use of LiCl in liquid media for improved selective enrichment of listerias is a logical step, particularly in media for the examination of foodstuffs which can contain high numbers of competitive flora. The first application of LiCl (3 g l^{-1}) was reported by Fraser and Sperber (1988). Later, Van Netten et al. (1989) incorporated LiCl at a level of 10 g l^{-1} in 'L-PALCAMY', a liquid enrichment medium for *Listeria* spp.

The 'Oxford' solid medium (Curtis et al. 1989) contains 1.5% LiCl, as does a medium described by Van Netten et al. (1988), concentrations which could render them hyperosmotic. This should be taken into account as a significant inhibitory factor.

Table 1 Positive and negative effects of lithium ions on microorganisms

General effect	Specific effect	Reference
On metabolism of halophiles	Na^+ sparing effect (various organisms)	Chan et al. 1979
	Inhibition of succinate oxidase and NADH dehydrogenase (marine bacterium 1055-1)	Ichikawa et al. 1981
	Stimulation/inhibition of autolysis	Sugahara et al. 1983
On cell wall synthesis	Production of unbalanced growth forms in *Listeria* spp.	Zigangirova et al. 1985
	Unbalanced growth in *Salmonella typhimurium*	Konstantinova et al. 1983
On metabolism and transport in Gram-negative organisms	Stimulation of proline transport in *Escherichia coli*	Chen et al. 1985
	Inhibition of aspartokinase in *E. coli*	Shaw et al. 1983
	Inhibition of melibiose transport in *E. coli*	Tanaka et al. 1980
	Stimulation of thiomethyl galactoside uptake in *E. coli*	Tsuchiya et al. 1978
	Inhibition of glycolysis and pyruvate kinase	Umeda et al. 1984
	Increased sensitivity of ATP-ase to dicyclohexylcarbodi-imide in *E. coli*	Feinstein and Fisher 1977
On Gram-positive organisms	Stimulation of sporulation in *Bacillus subtilis*	Warburg et al. 1985
	Reversal of F^+ enhancement of extracellular polysaccharide synthesis by *Streptococcus mutans*	Treasure 1981
On fungi	Stimulation of aflatoxin synthesis	Bhatnagar et al. 1979
	Growth inhibition	Kurita and Funabashi 1984
On yeasts	Growth inhibition	Takenishi and Takada 1984
	Increased transformation efficiency	Ito et al. 1984
Other non-specified	Growth inhibition of *Clavibacterium michiganensis*	Smidt and Vidaver 1986
	Growth inhibition of *Clostridium perfringens*	Bartsch and Walker 1982

Not only is the flora of food samples quite variable, but the food substrate may also have an effect on the inhibitory nature of media containing certain antibiotics (Beumer et al. 1988). The latter effect is particularly notable with samples containing proteins such as casein, which are thought to bind inhibitors such as acriflavine, thereby reducing the effective concentration in the medium (Chapter 3).

In view of the lack of information on the effects of LiCl, and the factors affecting its inhibitory properties, the following subjects were studied: growth of *Listeria* spp. and competing flora from foods in liquid media containing LiCl at different concentrations, the effect of the presence of high protein substrates on the inhibition of competing flora by LiCl, the nature of the competing food flora growing in the presence of LiCl, and the effect of different concentrations of nalidixic acid on the growth of *L. monocytogenes* and competitors.

MATERIALS AND METHODS

All solutions were prepared w/v. Antibiotics were added as appropiate volumes of filter sterilized stock solutions to give the absolute concentrations mentioned in mg l^{-1} of the final medium after autoclaving.

The effect of LiCl concentration and food substrate on the growth of *L. monocytogenes* and *Enterococcus faecalis*

Strains used for these experiments were from the Nestec culture collection and from the collection of the Agricultural University of Wageningen. They represented strains from natural, industrial and clinical sources.

As a control, tryptone soy broth supplemented with 6 g l^{-1} yeast extract was used (TSB). This medium was prepared by dissolving 30 g tryptone soy broth powder and 6 g of yeast extract in 900 ml distilled water, followed by autoclaving at 121°C for 15 min. Portions of 135 ml of this concentrated TSB were supplemented with 0.03, 0.3, 2.0 and 3% LiCl, added as 15 ml of filter sterilized solutions of appropiate dilutions of the salt in distilled water. The effect of high protein substrates was tested by addition of sterilized skimmed milk powder (SMP) at a level of 10% (w/v) to media containing 3.0% LiCl.

A loopful of the organism under test was transferred to 5 ml of TSB and incubated overnight at 30°C. Flasks containing 150 ml of the broth under investigation, pre-warmed to 30°C, were inoculated to give a level of about 10^2 cfu ml^{-1}. Broths were then

incubated statically at 30°C in a circulating water bath and samples taken periodically for enumeration.

Enumeration was carried out by spread-plating of decimal dilutions of samples taken at 0, 2, 4, 6, 9, 12, 18 and 24 h of incubation, onto tryptone soya agar (TSA) supplemented with 6 g l^{-1} yeast-extract. Plates were incubated at 37°C for 24 h. Growth curves were constructed for each experimental variant.

Competitive flora of meat and milk products, able to grow in the presence of LiCl

Milk and meat products were enriched in TSB + 2% LiCl (TSBLC) and/or TSB +15 mg l^{-1} acriflavine and 40 mg l^{-1} nalidixic acid (TSBTN) and the flora characterized.

Samples of Italian cheese (Cacetti), French soft cheese (Camembert and Tomme), feta cheese and raw ham (non-pasteurized, sliced) were purchased from a local supermarket. Twenty-five gram samples of each were mixed by stomaching with 225 ml of TSBLC (2%), prepared as described above.

Broths were incubated at 30°C for 24 h. After this time, a loopful from each broth was streaked on to blood agar, TSA and a modified McBride agar containing 0.3% LiCl (MMA+). Plates were incubated at 30°C for 24 h, after which, colonies of differing morphology were selected for identification. Isolated organisms were submitted to the usual identification scheme to genus level (Seeliger and Jones 1986).

Effects of LiCl and nalidixic acid on *Listeria* spp. and competing flora

To get to know whether non-*Listeria* microflora present in products does or does not grow in TSBLC, isolates obtained as above were tested for their ability to grow in TSBLC (2%) medium and TSBLC (2%) supplemented with 40 mg l^{-1} nalidixic acid (TSBLCN). In addition, 92 strains of *L. monocytogenes* (9: serotype 1/2a; 29: serotype 1/2b; 6: serotype 1/2c; 1: serotype 3a; 14: serotype 4b; 4: serotype '4'; 3: serotype '1'; 26: not serotyped), and 54 strains of *L. innocua* and one strain of *L. seeligeri* from our collections were tested for growth in TSBLC.

A loopful of an overnight culture of each competitor or *Listeria* strain in TSB medium was inoculated into 200 µl of TSBLC (2%) as a control and 200 µl of TSBLCN in wells of microtitre plates. These were incubated at 30°C for 24 h and then examined for visible growth.

Effect of nalidixic acid concentration on growth of *L. monocytogenes*

TSB was prepared with different concentrations of nalidixic acid ranging from 0 to 80 mg l^{-1}. Quantities of 100 ml were inoculated with *L. monocytogenes* (about 10^3-10^4 cfu ml^{-1}) These were incubated at 30°C for 16 h. Enumeration was carried out as previously described and growth curves were constructed.

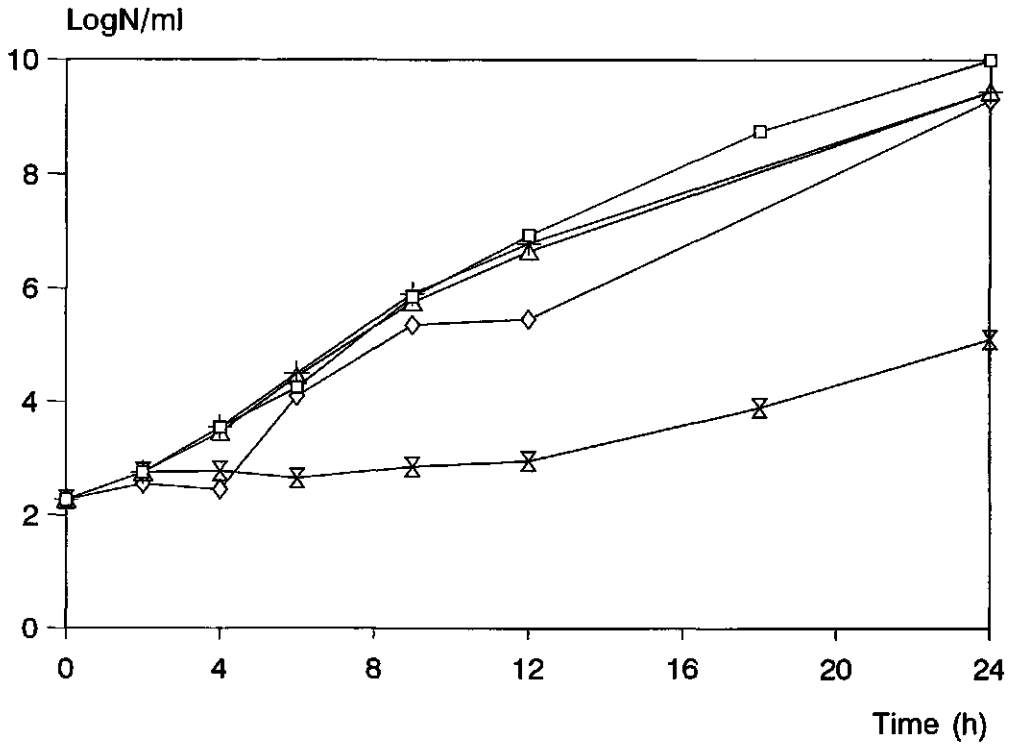

Fig.1 Effect of LiCl concentration on growth of *Listeria monocytogenes* CMB in tryptone soya broth (□) 0% LiCl, (+) 0.03% LiCl, (△) 0.3% LiCl, (◇) 2% LiCl, (⧗) 3% LiCl

Effect of acriflavine and LiCl on growth of *L. monocytogenes*

To investigate whether LiCl and acriflavine in combination had an inhibitory effect on *L. monocytogenes*, the organism was grown in TSB containing 0.3% and 2% LiCl plus 12 mg l^{-1} acriflavine. A growth curve was obtained as previously described.

RESULTS

Effects of LiCl concentration on growth of *L. monocytogenes* CMB and *E. faecalis*

Figs 1 and 2 show the effect of LiCl concentration on the growth of these organisms.

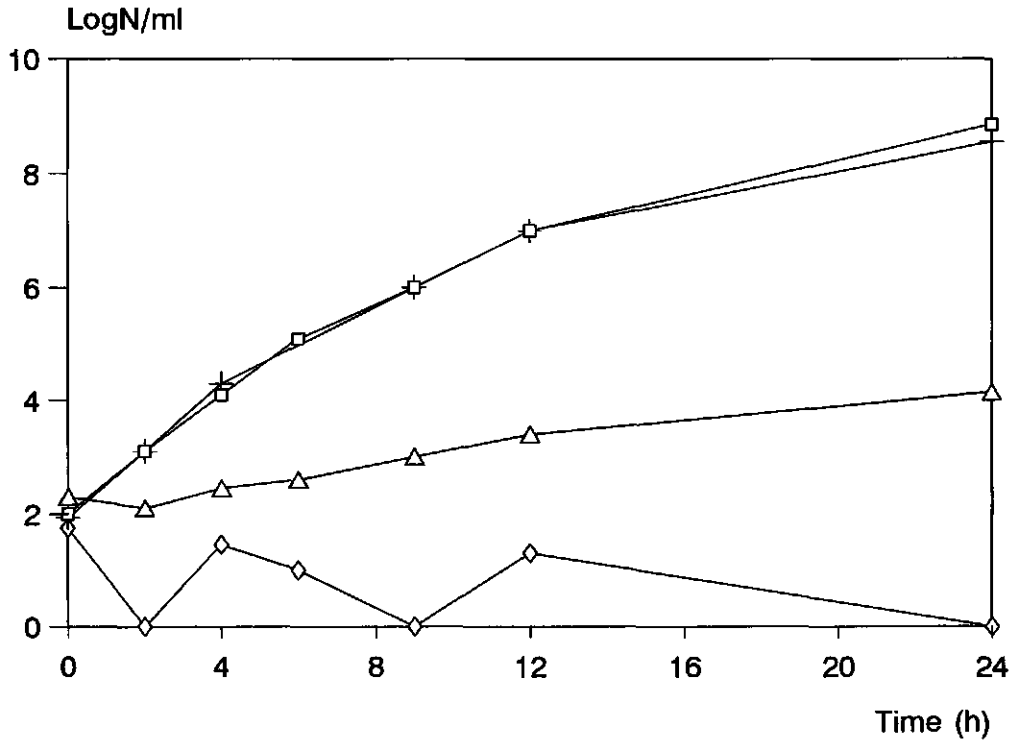

Fig.2 Effect of LiCl concentration on growth of *Enterococcus faecalis* in tryptone soya broth (□) 0% LiCl, (+) 0.03% LiCl, (△) 0.3% LiCl, (◇) 2% LiCl

From an initial inoculum level of 2.3 (log number of cells) ml^{-1}, *L. monocytogenes* achieved a final level of 9.8 ml^{-1} after 24 h in TSB. Growth in the presence of 0.03 and 0.3% LiCl followed essentially similar kinetics although the final levels achieved (9.6 ml^{-1}) were slightly, but not significantly less than the control. In the presence of 2%

LiCl, the growth curve assumed a slightly flatter slope with a lag phase of approximately 3 h and a plateau between 9 and 12 h. However, the final number of organisms after 24 h was 9.4 ml^{-1}, which was similar to that achieved in the presence of 0.3% LiCl. The presence of 3% LiCl was definitely inhibitory. Numbers increased only

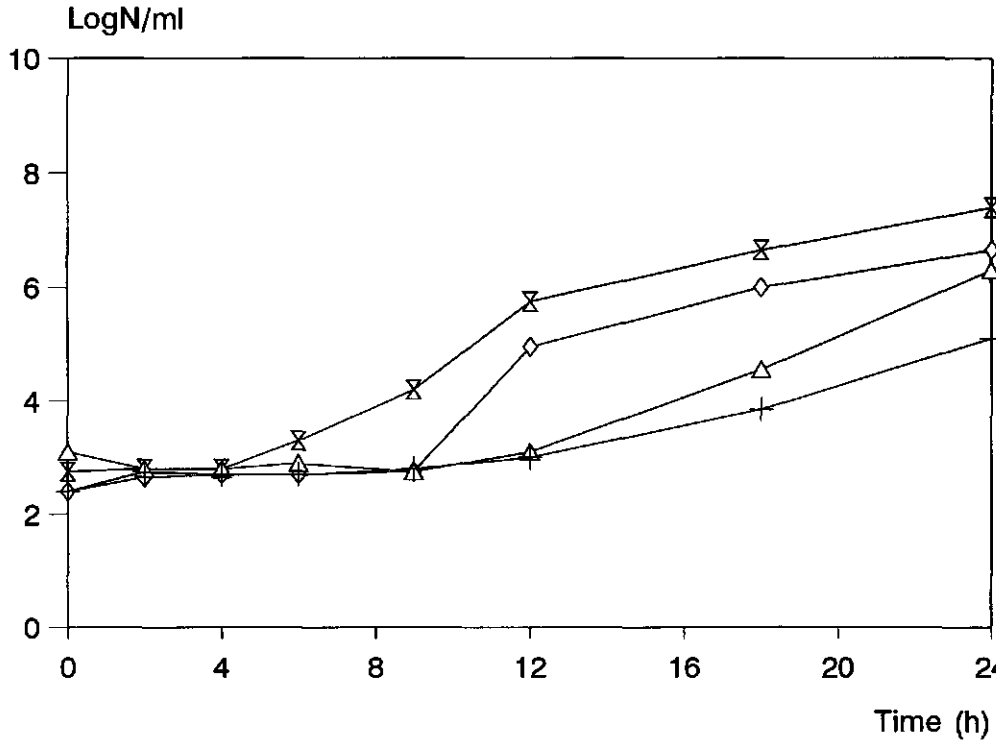

Fig.3 Effect of skim milk powder (SMP) on growth of *Listeria monocytogenes* and *Listeria innocua* in tryptone soya broth with 3% LiCl (+) *Lm* 3% LiCl, (▵) *Li* 3% LiCl, (◇) *Lm* 3% LiCl+SMP, (⧗) *Li* 3% LiCl+SMP

slightly over the first 12 h to 3.0 ml^{-1}, after which a slow increase to 5.1 ml^{-1} occurred at 24 h. A minor difference between the effect of 1 and 2% LiCl was observed (data not shown).

E. faecalis, like *L. monocytogenes*, grew well in TSB with a typical sigmoid curve and attained a level of 8.9 ml^{-1} after 24 h (Fig. 2). The presence of 0.03% LiCl had little effect on the final number attained (8.6 ml^{-1}) and did not change the shape of the curve. However, 0.3% LiCl resulted in a severe inhibition (4.2 ml^{-1} after 24 h) and 2% LiCl resulted in death of the original inoculum.

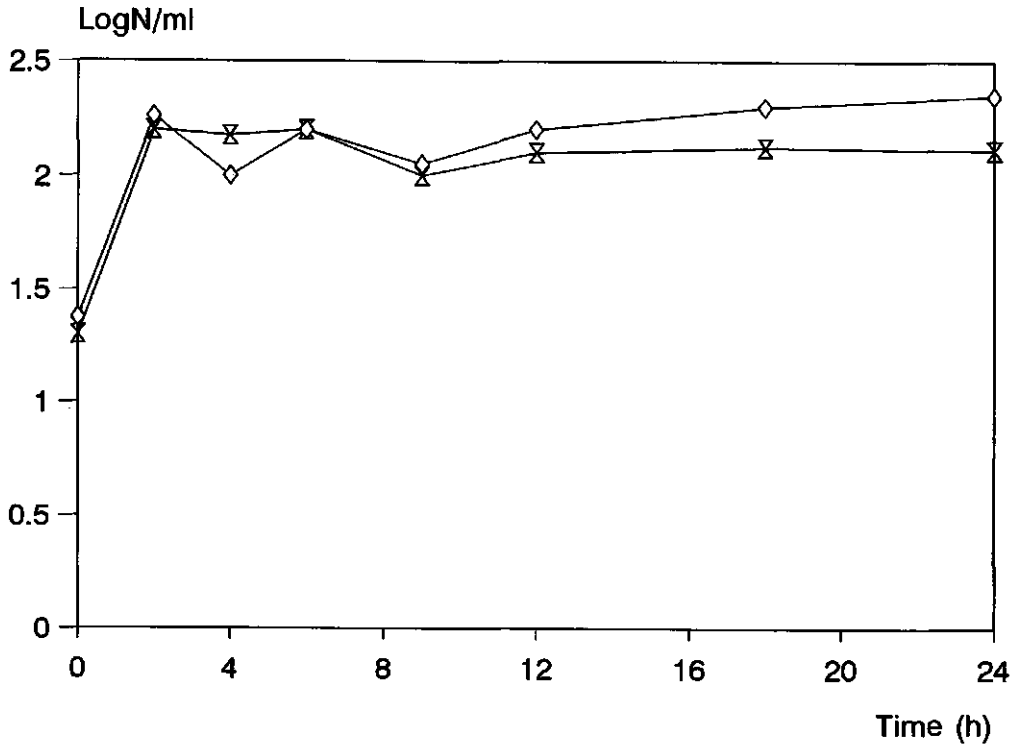

Fig.4 Effect of skim milk powder (SMP) on growth of *Enterococcus faecalis* in tryptone soya broth with LiCl (◇) 3% LiCl, (⧖) 3% LiCl+SMP

Fig. 4 shows the effect of SMP addition on the growth of *E. faecalis* in the presence of 2 and 3% LiCl. No significant effect on inhibition was observed.

Effect of substrate addition on the growth of *L. monocytogenes* CMB, *L. innocua* and *E. faecalis* in LiCl-containing media

In the presence of SMP, the inhibitory effect of 3% LiCl on both *L. innocua* and *L. monocytogenes* was alleviated significantly (Fig. 3). In the presence of LiCl (3%), *L. innocua* followed the same lag phase course as for *L. monocytogenes* up to 12 h. After this time, growth of *L. innocua* followed a steeper curve and reached about 6.0 ml^{-1} after 24 h compared to only 5.0 ml^{-1} for *L. monocytogenes*. In the presence of SMP, the lag phase for *L. innocua* lasted around 4 h only. Growth followed an exponential course until 12 h, after which a flattening-off was seen. A final level of around 7.5 ml^{-1} was

Table 2 Competitive microflora isolated from TSB+2% lithium chloride (TSBLC) medium

Product	Enrichment	Solid medium	# isolates	Identity (#)
Camembert	LiCl	blood agar	12	*Streptococcus* spp. (7) *Staphylococcus* sp. (1) *Pseudomonas* sp. (1) *Bacillus* spp. (3)
	TSBYETN	blood agar	3	*Streptococcus* spp. (3)
Feta	LiCl	TSA	2	*Proteus vulgaris* (1) *Citrob. freundii* (1)
		MMA+	4	*Staphylococcus* spp. (4)
Cacetti	LiCl	TSA	9	*Bacillus* spp. (7) *Staphylococcus* sp. (1) *Hafnia alvei* (1)
		MMA+	9	*Staphylococcus* spp. (8) *Bacillus* sp. (1)
Tomme	LiCl	TSA	2	*Citrobacter freundii* (1) *Streptococcus lactis* (1)
Ham	LiCl	TSA	4	*Bacillus* spp (3) *Staphylococcus* sp. (1)
		MMA+	5	*Staphylococcus* spp. (5)

reached after 24 h. *L. monocytogenes* showed a longer lag phase of 9 h after which growth occurred rapidly, reaching approximately 5.0 ml^{-1} at 12 h. After this, however, the curve flattened and a level of approximately 6.5 ml^{-1} was achieved after 24 h.

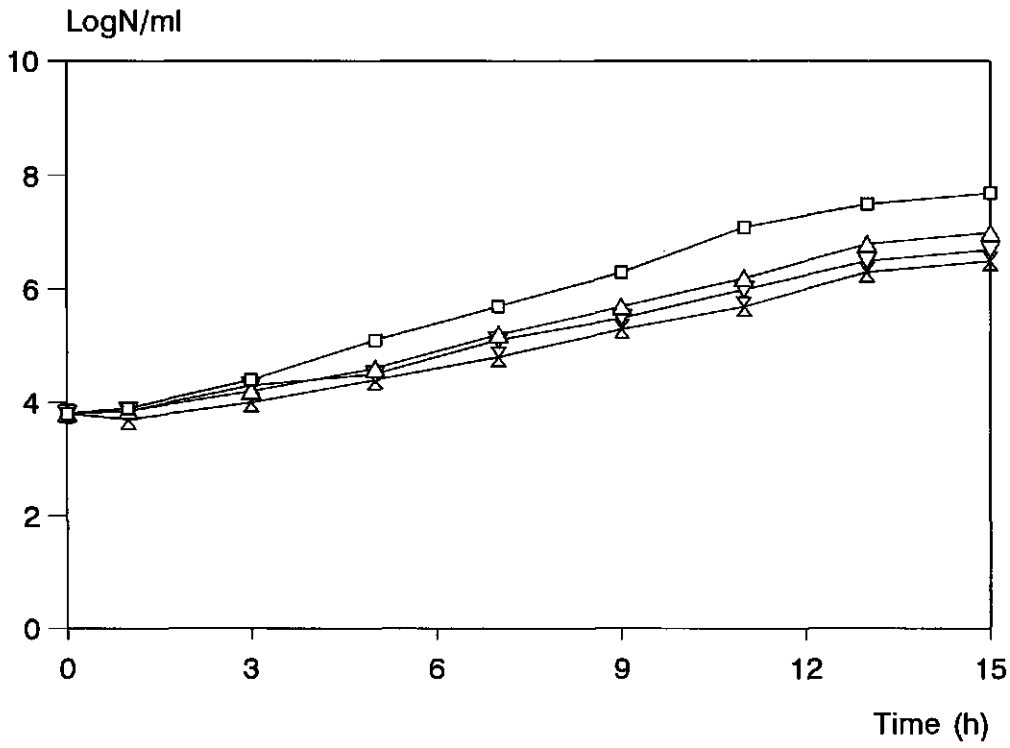

Fig.5 Effect of nalidixic acid concentration on *Listeria monocytogenes* (serotype 4b) in tryptone soya broth (□) 0 $\mu g\ ml^{-1}$, (△) 40 $\mu g\ ml^{-1}$, (▽) 60 $\mu g\ ml^{-1}$, (⧗) 80 $\mu g\ ml^{-1}$

Competitive flora growing in the presence of 2% LiCl

Table 2 shows the results of the study of microorganisms present in TSBLC (2%) medium that were able to grow both on MMA+, blood agar and TSA. From a total of 18 isolates on MMA+, 17 were identified as *Staphylococcus* spp. The remaining isolate

was a *Bacillus* sp. A total of 32 isolates was obtained from non-selective media, of which 11 were identified as *Streptococcus* spp., 3 *Staphylococcus* spp., 13 *Bacillus* spp., 4 *Enterobacteriaceae* and one *Pseudomonas* sp.

Growth of *Listeria* spp. and isolated competing flora in TSBLC and TSBLCN

All strains of *Listeria* tested, grew in TSBLC and TSBLCN, giving highly turbid suspensions after 24 h at 30°C.

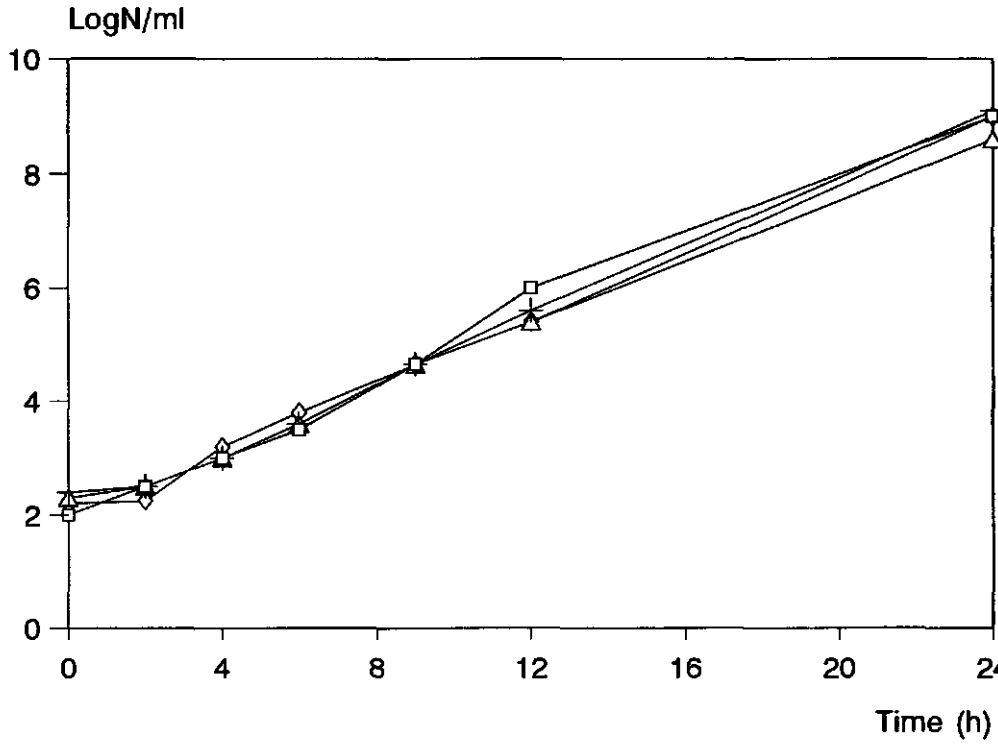

Fig.6 Effect of lithium chloride on growth of *Listeria monocytogenes* in tryptone soya broth containing acriflavine (12 mg l^{-1}) (□) 0% LiCl, (+) 0.03% LiCl, (△) 0.3% LiCl, (◇) 2% LiCl

Of the isolates of competitors obtained from TSBLC and TSBYETN enrichments, all, apart from the 22 *Staphylococcus* spp. strains, were inhibited in TSBLCN medium, and all, except the *Bacillus* spp., grew in TSBLC.

Effect of nalidixic acid concentration on growth of *L. monocytogenes*

Although nalidixic acid tended to inhibit the *Listeria* strains used (Fig. 5), this inhibition was not significantly different between 40 and 60 mg l^{-1}, since there was no apparent lag phase and the growth rate was not much affected. Concentrations above 60 mg l^{-1} tended to further reduce the growth rate.

Effect of LiCl and acriflavine on growth of *L. monocytogenes*

The growth of *L. monocytogenes* was not inhibited by the presence of these two compounds simultaneously (Fig. 6), in a way that might preclude their use in an enrichment procedure.

DISCUSSION

Lithium chloride is an efficient inhibitor of a relatively large variety of microorganisms present in foods. If used at concentrations of 2% or less, it has no significant effect on *L. monocytogenes* inocula grown under optimum conditions. Although the strain of *E. faecalis* used for plotting growth curves was completely inhibited by LiCl, it can be seen from the isolates from foods (Table 2) that perhaps not all streptococci will be inhibited in enrichments and indeed even some *Enterobacteriaceae* may grow. However, the addition of nalidixic acid seems to inhibit quite a number of these, since the only organisms capable of growing in the TSBLCN medium were staphylococci.

The effect of substrates such as cheese on growth of *Listeria* in enrichment media has already been studied by Jung (1987) and Beumer et al. (1989), who have shown that addition of cheese protein can render acriflavine inactive towards streptococci. The inhibitory effect of 3% LiCl on both organisms was diminished significantly by skim milk powder (SMP). Although the final growth rate for both was still somewhat less than that in the presence of 2% LiCl, the final numbers achieved after 24 h were in the order of log 6 for *L. monocytogenes* and log 7.5 for *L. innocua*. SMP caused no relief of inhibition on *E. faecalis* in the presence of LiCl.

It is possible that relief of inhibition by SMP is not due to a simple binding of Li^+ or that the mechanism of action of LiCl on streptococci is different from that on listerias. This is supported by the fact that the concentrations required to inhibit listerias are about tenfold higher than those which inhibit some other organisms such as streptococci. Further studies of the effect of LiCl are necessary to elucidate the mechanism of action, but it could be hypothesised that SMP might contain compatible solutes which could provide a certain protection against an osmotic effect of 3% LiCl.

The results presented in this study strongly suggest that LiCl is as good an inhibitor of competitive flora as acriflavine in enrichment media for listerias and could be used to replace or supplement the antibiotic. It does not inhibit listerias significantly if used at concentrations up to 2%, whereas acriflavine can be inhibitory to some strains of *L. monocytogenes* at concentrations required to effectively inhibit competing flora in currently recommended media (Beumer et al. 1988). Furthermore, the inhibition of competing organisms by LiCl seems not to be dependent on the type of substrate used in the same way as is inhibition by acriflavine. There is therefore a good case to be made for the simultaneous presence of these two inhibitors. LiCl used in combination with nalidixic acid inhibits many competing organisms present in foods, thus rendering it easier to detect *Listeria* on solid media. Since there is a certain effect of nalidixic acid on the growth of *Listeria* (Fig. 6), there is certainly a case to be made for lowering the concentration further to 20 mg l^{-1}, as already used by Lee and McClain (1988). Based on the above findings, further comparative studies are required to judge the relative merits of different enrichments to optimise the recovery of *Listeria* spp. But one should realize that detection of *L. monocytogenes*, both with media based on LiCl and the media used in FDA and USDA protocols, may be hampered by faster growing listerias.

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3

THE EFFECT OF ACRIFLAVINE AND NALIDIXIC ACID ON THE GROWTH OF *LISTERIA* SPP. IN ENRICHMENT MEDIA

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SUMMARY

The use of acriflavine in enrichment media for *Listeria* spp. has both direct and indirect effects on the isolation of *Listeria monocytogenes*. Increasing acriflavine concentrations affect both lag time and generation time of *L. monocytogenes*, whereas hardly any effect is observed on *L. innocua*. Because acriflavine binds to protein in the samples, a decrease in acriflavine activity results. This lesser activity may result in a better growth of *L. monocytogenes*. At low pH-values (pH <5.8) more acriflavine is bound, but growth promoting effects are limited, because growth of this pathogen is restricted at low pH. On account of this, one may expect that enrichment protocols employing low acriflavine concentrations with an adequate buffer, favour the isolation of *L. monocytogenes*. Because comparative studies have paid no attention to the ratio *L. monocytogenes* to other *Listeria* spp., virtually nothing is known about the inferior detection of *L. monocytogenes*. Previous comparative studies combined with the results of this work indicate stongly that during enrichment procedures, other listerias or competitive microorganisms may mask the presence of this pathogen. For that reason, in enrichment protocols for the detection of *L. monocytogenes*, it is worthwhile introducing an isolation medium that facilitates identification of *L. monocytogenes* in the presence of high numbers of other listerias.

3

INTRODUCTION

In the enrichment procedures proposed by US Food and Drug Administration (FDA) (Lovett et al. 1987) and the US Department of Agriculture (USDA) acriflavine, nalidixic acid and cycloheximide are used as selective agents (McClain and Lee 1988).

Acriflavine inhibits RNA synthesis and mitochondriogenesis (De Vries and Kroon 1970, Meyer et al. 1972). It is commonly used in enrichment and enumeration protocols, often in combination with various other selective agents such as potassium thiocyanate, polymyxin B-sulphate, nalidixic acid and cycloheximide. The proposed concentration of acriflavine in the media varies from 10−25 mg l^{-1} (Prentice and Neaves 1988). The use of acriflavine in media for the isolation of *Listeria* spp. was first described by Ralovich et al. (1971). They concluded that *Listeria monocytogenes* multiplied well when acriflavine (40 mg l^{-1}) was added to agar plates whereas growth of Gram-positive cocci was suppressed. In the same year Bockemühl et al. (1971) published results of their studies with 12 acridine dyes as selective agent in media for the isolation of *L. monocytogenes*. With three of these (xanthacridin, neutral acriflavine and proflavinehemisulphate) a pronounced inhibition of enterococci was observed. Neutral acriflavine was defined as 2,8-diamino-10-methylacridiniumchloride, but is now considered to be a mixture of the hydrochlorides of 3,6-diamino-10-methylacridine and 3,6-diaminoacridine. It is known as acriflavine HCl or acriflavine in the Merck Index (Budvari et al. 1989).

Nalidixic acid inhibits the DNA synthesis of cells and has already been used by Beerens and Tahon-Castel (1966). Following this report, nalidixic acid has been applied in various formulations of enrichment and isolation media to inhibit the growth of Gram-negative microorganisms. In the majority of these media, nalidixic acid is combined with other inhibitory agents (Klinger 1988, Prentice and Neaves 1988). One such agent is cycloheximide (actidione) which inhibits protein synthesis in eukaryotic cells by binding to 80S ribosomal RNA (Obrig 1971). When included in enrichment or isolation media it prevents growth of most yeasts and moulds.

It is known that microorganisms of the same genus are not equally affected by inhibitors used in selective enrichment media. In procedures where all species of a genus are considered to be pathogenic such as *Salmonella* only epidemiologically false information may be obtained. However, in case of detecting *L. monocytogenes*, the only *Listeria* species which is pathogenic in humans, faster growing listerias may mask the presence of this pathogen. Numerous studies have indicated that *L. innocua* can grow

faster than *L. monocytogenes*, notably in the non-selective medium brain heart infusion broth (BHIB) (Due and Schaffner 1993). Nevertheless, Petran and Swanson (1993) concluded from their experiments with clinical and milk isolates of *L. monocytogenes* and *L. innocua*, that growth rates of the two species in non-selective trypticase soy broth plus 0.6% yeast extract (TSBYE) or cheese sauce were comparable. However, in selective enrichment media: University of Vermont broth (UVM, BBL) and Fraser Broth (Difco), *L. innocua* reached significantly higher levels than *L. monocytogenes* strains, approximately 1 log unit and 1.9 log unit, respectively. Similar results were obtained in impedance studies in *Listeria* enrichment broth (Oxoid) with strains isolated from raw cows' milk (MacDonald and Sutherland 1994). The authors also reported differences in generation times and suggested that these are probably due to variations in sensitivity to enrichment broth components and incubation procedures because in non-selective tryptone soya broth (TSB), generation times were similar for both species. In experiments with samples inoculated with both *L. monocytogenes* and *L. innocua*, the latter was recovered from all samples, whereas the recovery rate of *L. monocytogenes* varied from 0−92%, depending on the ratio of this pathogen to *L. innocua* directly after inoculation (from 1:1−1200:1). Thus, it is clear that the incidence of *L. monocytogenes* may well be underestimated, because enrichment appears to favour growth of non-pathogenic *L. innocua* (Curiale and Lewus 1994).

Considering the lack of information on the actual effect of inhibitors employed in *Listeria* enrichment media, it is worth while to examine and compare the growth of different *Listeria* spp. in the presence of various concentrations of acriflavine and nalidixic acid. In addition, the presence of food components on the inhibitory activity and the stability of these selective agents was investigated.

MATERIALS AND METHODS

Bacterial strains

The *Listeria* strains used in this study were: *L. innocua* serotype 6A, isolated from silage (1) and from soft cheese (2,3). *L. innocua* serotype 6B, isolated from silage (4) and from soft cheese (5,6). *L. monocytogenes* serotype 1/2A, isolated from soft cheese (7,8) and from a patient (9). *L. monocytogenes* serotype 1/2B, isolated from soft cheese (10,11) and from a patient (12). *L. monocytogenes* serotype 4B, isolated from soft cheese (13,14), from a patient (15) and from meat products (II,C).

Bacillus subtilis ATCC 6633 and *Klebsiella pneumoniae* LUW were used in the experi-

ments to determine the antimicrobial activity of acriflavine and nalidixic acid. Strains were stored at −20°C in TSBYE (Oxoid)+20% glycerol.

Growth experiments

Growth experiments were conducted in TSBYE (100 ml in 200 ml flasks). Just before use, filter sterilized solutions of acriflavine (Fluka 01675) and/or nalidixic acid (Winthrop) were added at various concentrations. The media were inoculated adding appropriate dilutions of overnight (22−24 h, 30°C) TSBYE cultures in peptone physiological salt solution (PPSS, composition: NaCl (8.5 g l^{-1}) and neutralized bacteriological peptone (Oxoid L34 1 g l^{-1}). The inoculum level (about 10^3 listerias ml^{-1}) was checked by surface streaking on TSBYE (with 1.2% agar, Oxoid L13 added). Flasks were incubated in a thermostatically controlled water-bath at 30°C. During incubation 1 ml samples (or appropriate dilutions in PPSS) were removed regularly and plated in duplicate on TS-YEA-plates, which were incubated at 30°C for two days.

All plates were examined using a modified Henry's illumination (Larsen 1969) by means of a stereo microscope (Olympus VMZ) with darkfield illumination (Olympus ILLD). Blue to greyish-blue colonies were considered to be *Listeria*.

In growth experiments where the effects of competing microorganisms was undesirable, irradiation (10 kGy) was applied (Provo, Wageningen, The Netherlands) in the case of soft cheese (Camembert), poultry meat and minced meat. Growth curves were generated from the experiment data using the modified Gompertz equation. The growth rate and lag time were calculated using a growth-curve fitting program (Zwietering et al. 1990).

Binding of acriflavine to proteins

Acriflavine solution was filter sterilized and added to TSBYE to final concentrations of 3, 6, 9, 12, 15, 18 and 21 mg l^{-1}. Samples (2 ml) were removed, and 100 µl trichlor acetic acid (TCA, Merck 807.0250, 1.2 g ml^{-1} distilled water) was added to precipitate proteins. The solutions were centrifuged for 20 min at 14 000 g. Removal of residual protein was achieved by addition of a further 100 µl of TCA to the supernatant followed by centrifugation for 30 min at 14 000 g. This was repeated when necessary. Absorbance of the resulting supernatant was measured at 440 nm and the values obtained were used to construct a calibration curve (R=0.99).

In enrichment pocedures with food (minced meat, poultry meat, pâté, brie, milk and white cabbage) binding of acriflavine to proteins in the food was determined as described above.

The effect of various pH-values was determined in (1:4) suspensions of pâté with citric acid-phosphate buffers (pH 4.1, 4.4, 4.7, 4.9, 5.1, 5.3, 5.4, 5.6, 5.8, 6.2, 6.4, 7.0 and 7.5) according to McIlvaine. Binding of acriflavine to proteins was calculated by conversion of the absorption values with the calibration curve.

Antimicrobial activity of acriflavine and nalidixic acid

Antimicrobial activity of acriflavine in TSBYE and TSBYE combined with food suspensions (cheese, minced meat, chicken meat) was assessed by adding 20 μl (in duplicate) of the centrifuged enrichment broth to wells (Ø 6 mm) in TS-YEA plates, seeded with *B. subtilis* (sensitive to acriflavine). These plates were prepared by adding 1 ml of overnight cultures (24 h, 30°C) of *B. subtilis* to 100 ml TS-YEA. After incubation for 16–24 h at 30 °C inhibition zones were measured and compared with wells to which PPSS had been added as a negative control. Antimicrobial action of nalidixic acid was determined in the same way using TS-YEA plates seeded with an overnight culture (24 h, 37°C) of *K. pneumoniae*.

RESULTS

From the results presented it becomes clear that increasing acriflavine concentrations affect both lag time and generation time of *L. monocytogenes* II in TSBYE (Fig. 1). *L. monocytogenes* C was unable to grow in presence of 15 mg l^{-1} acriflavine (Fig. 2). The effect of acriflavine (15 mg l^{-1}) on other *Listeria* spp. is presented in Table 1. The inhibitory effect seemed more obvious for *L. monocytogenes* serotype 4b than for serotypes 1/2a and 2b, whereas hardly any effect was observed on *L. innocua*. Presence of food components reduced the inhibition as is shown for minced meat and poultry (Fig. 2) and for cheese (Table 1). However, this effect could not be confirmed statistically.

Nalidixic acid at concentrations of 20 and 40 mg l^{-1} had little effect on the growth of *Listeria* spp. in enrichment media. When the concentration was increased to 100 mg l^{-1} the growth rate was reduced for all species. Addition of irradiated cheese to TSBYE (1:9) with nalidixic acid (40 mg l^{-1}) did not result in stimulation of growth (Table 2). The enhanced growth of *L. monocytogenes* in enrichment media after addition of food products suggests that food components interact with acriflavine and reduce its inhibi-

tory capacity. In suspensions of the food with TSBYE (1:9) it was observed that, depending on the type of food and the concentration of acriflavine present, 19–79% of this inhibitor was bound to protein. After incubation for 24 h at 30° C these values increased to 43–100% (Table 3).

Interestingly, the rather low values for bound acriflavine in white cabbage suspensions (low protein content) increased up to 100% after 48 h incubation. This suspension also showed the greatest decrease in pH (data not shown), this might indicate that binding of acriflavine to proteins is also pH-dependent. This is confirmed by the results of the experiments presented in Fig. 3. Binding of acriflavine increased below pH 5.8.

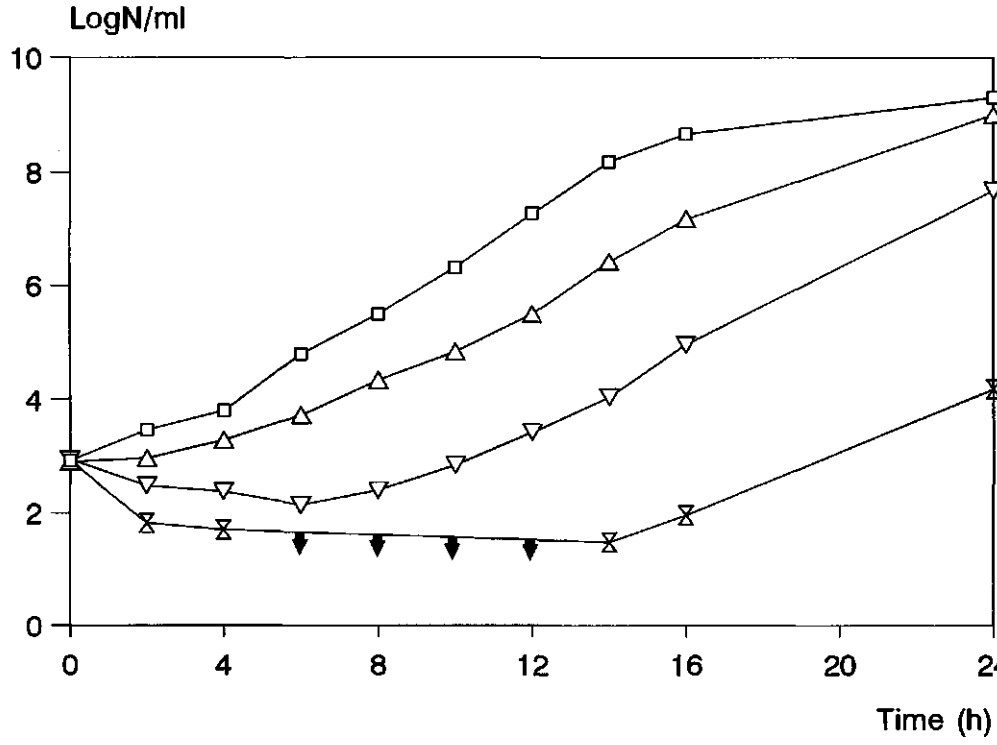

Fig.1 Effect of acriflavine on the growth of *Listeria monocytogenes* 4B (II) in tryptone soya broth + 0.6% yeast extract (TSBYE) at 30°C (□) 0 mg l^{-1} acriflavine, (△) 5 mg l^{-1}, (▽) 10 mg l^{-1} and (⧖) 15 mg l^{-1}

The binding of acriflavine will result in loss of inhibitory activity of this agent. This is shown in Table 4 by the reduction in zones of inhibition in TS-YEA/BS plates for both BLEB and UVM-1 and in suspensions of food (1:9) with these enrichment media. No significant decrease in inhibitory activity of nalidixic acid could be determined.

Table 1 Lagphase (h) and specific growth rate (h^{-1}) of *Listeria* spp. at 30°C in tryptone soya broth + 0.6% yeast extract (TSBYE), in TSBYE with acriflavine (15 mg l^{-1}) added (TSBYE-15) and in TSBYE-15 with gamma sterilized cheese (TSBYE-15GC)

	Lag phase (h)			Specific growth rate (h^{-1})		
***Listeria* spp.**	**TSBYE**	**TSBYE-15**	**TSBYE-15GC**	**TSBYE**	**TSBYE-15**	**TSBYE-15GC**
Listeria innocua						
1, 6A	1.4 ± 0.9[a]	1.5 ± 1.0		0.87 ± 0.12	0.85 ± 0.12	
2, 6A	1.2 ± 1.2	1.1 ± 1.2		0.76 ± 0.12	0.77 ± 0.12	
3, 6A	2.1 ± 0.9	2.3 ± 0.9	1.5 ± 1.1	0.88 ± 0.12	0.86 ± 0.12	0.86 ± 0.14
4, 6B	2.4 ± 0.4	2.5 ± 0.4		1.06 ± 0.08	1.09 ± 0.07	
5, 6B	1.7 ± 0.9	1.7 ± 1.0		0.87 ± 0.12	0.85 ± 0.12	
6, 6B	1.5 ± 0.7	1.7 ± 0.8	1.5 ± 0.7	1.03 ± 0.12	1.04 ± 0.14	1.06 ± 0.13
Listeria monocytogenes						
7, I/2A	2.6 ± 0.3	2.7 ± 0.9		1.11 ± 0.07	0.90 ± 0.12	
8, I/2A	2.2 ± 0.9	4.0 ± 0.7		0.86 ± 0.12	0.80 ± 0.10	
9, I/2A	2.2 ± 0.6	4.9 ± 0.9	2.3 ± 1.0	1.15 ± 0.12	0.90 ± 0.16	1.00 ± 0.16
10, I/2B	2.8 ± 0.4	4.3 ± 0.6		0.95 ± 0.07	0.83 ± 0.08	
11, I/2B	3.0 ± 0.4	3.7 ± 0.5		1.08 ± 0.07	0.94 ± 0.08	
12, I/2B	3.3 ± 0.3	4.6 ± 0.6	3.4 ± 0.4	1.16 ± 0.06	0.92 ± 0.10	1.00 ± 0.06
13, 4B	3.1 ± 0.4	5.4 ± 0.5		0.95 ± 0.06	0.86 ± 0.09	
14, 4B	2.7 ± 0.4	4.3 ± 0.6		1.05 ± 0.07	0.90 ± 0.10	
15, 4B	3.2 ± 0.2	8.0 ± 0.5	4.0 ± 0.5	1.02 ± 0.04	0.89 ± 0.15	0.91 ± 0.08

[a] ± = 95% confidence limits

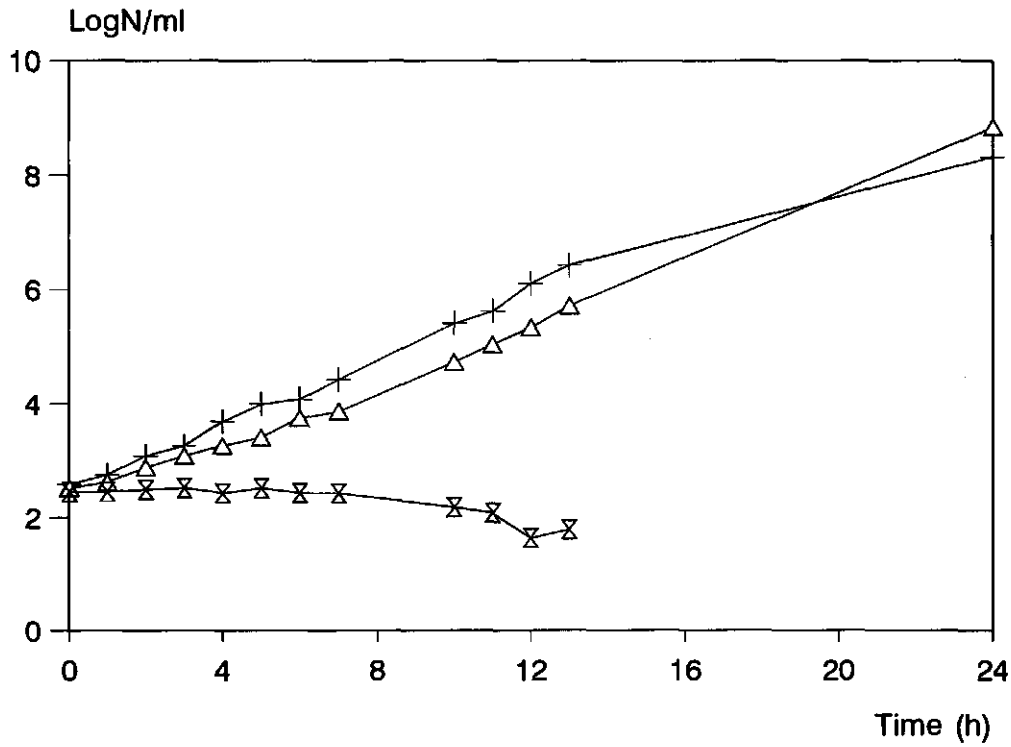

Fig.2 Growth of *Listeria monocytogenes* 4B (C) in tryptone soya broth + 0.6% yeast extract (TSBYE) with 15 mg l^{-1} acriflavine (TSBYE-AF) (I) and in (1:9) suspensions of poultry meat (△) and minced meat (+) with TSBYE-AF at 30°C

DISCUSSION

From the results presented in Figs 1 and 2 and Table 1 it becomes clear that acriflavine had an inhibitory effect on the growth of *L. monocytogenes* in TSBYE, manifested by an increase in lag time. The observed increase in generation time in selective enrichment media agrees with the findings of other workers (Petran and Swanson 1993, MacDonald and Sutherland 1994, Curiale and Lewus 1994), and may be explained by the inhibitory activity of the selective agents on *L. monocytogenes*. In enrichment procedures the food

material investigated may reduce this inhibition as demonstrated with minced meat and chicken meat (Fig. 2) and cheese (Table 2). Because the food samples were irradiated before mixing with TSBYE an effect of the functional flora (lactococci) in cheese or competitive microorganisms (minced meat, chicken meat) can be excluded. In the binding experiments, it was shown that acriflavine can bind to proteins resulting in a decrease of up to 60% of the initial concentration, depending on the pH (Table 3, Fig. 3). The type of protein (availability of carboxyl-groups) and the structure of the food (liquid, minced or cut in pieces) determine the amount of acriflavine that will be bound.

Table 2 Effect of different nalidixic acid concentrations on lag phase (h) and growth rate (h^{-1}) of *Listeria* spp. in tryptone soya broth + 0.6% yeast extract (TSBYE) and in sterile cheese suspension (1:9) in TSBYE with nalidixic acid added in a concentration of 40 mg l^{-1}

	Nalidixic acid concentrations in TSB YE (mg l^{-1})						
***Listeria* spp.**	**0**	**20**	**40**	**60**	**80**	**100**	**40[a]**
Listeria innocua 2, 6A							
Specific growth rate (h^{-1})	0.76	0.75	0.77	0.70	0.64	0.62	0.78
Lag phase (h)	1.4	1.4	1.5	1.5	1.5	1.6	1.5
Listeria innocua 6, 6B							
Specific growth rate (h^{-1})	0.88	0.86	0.89	0.84	0.78	0.66	0.87
Lag phase (h)	2.1	2.0	2.2	2.0	2.4	2.4	2.2
Listeria monocytogenes 9, 1/2A							
Specific growth rate (h^{-1})	0.86	0.86	0.84	0.79	0.72	0.65	0.84
Lagphase (h)	2.5	2.5	2.5	2.6	2.6	2.6	2.5
Listeria monocyogenes 11, 1/2B							
Specific growth rate (h^{-1})	1.10	1.12	1.10	0.98	0.92	0.76	1.11
Lagphase (h)	3.2	3.2	3.4	3.4	3.4	3.4	3.4
Listeria monocytogenes 14, 4B							
Specific growth rate (h^{-1})	1.10	1.08	1.06	0.95	0.87	0.79	1.01
Lagphase (h)	3.0	3.0	3.0	3.3	3.3	3.3	3.0

[a] 10% (w/v) sterile cheese suspended in TSBYE with 40 mg l^{-1} nalidixic acid

Binding of acriflavine to proteins will result in a decrease of activity as was demonstrated in the bio-assay with *B. subtilis* (Table 3). The reduced activity of acriflavine, through interaction with food, may result in a better growth of *L. monocytogenes* (Fig. 2, Table 2), although this is also dependent on the pH of the enrichment medium. At low pH-values (pH < 5.8) acriflavine binding is increased, nevertheless a potential for increased growth of the pathogen is reduced by low pH. On account of this, one might

Table 3 Percentage of acriflavine bound to proteins in enrichment procedures of food products in tryptone soua broth + 0.6% yeast extract (TSBYE) direct after stomacher treatment and after 24 and 48 h at 30°C

Product	**Concentration of**	**Acriflavine bound to protein (%)**		
(g protein 100 g^{-1})	acriflavine (mg l^{-1})	**t=0 h[a]**	**t=24 h**	**t=48 h**
Minced meat	12	60	74	61
(15)	15	57	65	57
	20	55	64	55
Poultry meat	12	43	58	59
(20)	15	46	61	60
	20	39	55	58
Pâté	12	45	59	60
(13)	15	51	60	62
	20	50	76	60
Brie	12	79	59	59
(22)	15	77	65	65
	20	64	68	67
Milk	12	71	69	67
(3.5)	15	53	49	52
	20	46	43	43
White cabbage	12	45	93	100
(1.7)	15	29	67	73
	20	19	57	59

[a] after stomacher treatment

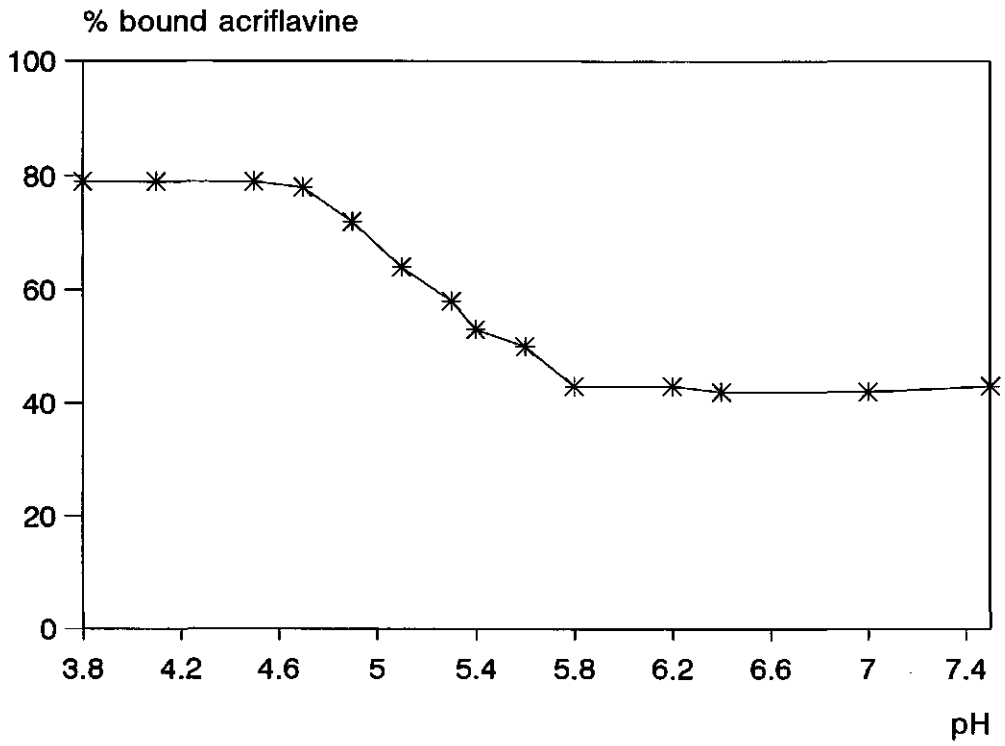

Fig.3 Binding of acriflavine to proteins in (1:4) suspensions of pâté with citric acid-phosphate buffers (pH 4.1, 4.4, 4.7, 4.9, 5.1, 5.3, 5.4, 5.6, 5.8, 6.2, 6.4, 7.0 and 7.5) according to McIlvaine

expect that enrichment procedures using low acriflavine concentrations and with an adequate buffer favour the isolation of *L. monocytogenes*. Nalidixic acid had no significant effect on growth of *L. monocytogenes* at the concentrations applied in enrichment media. The inhibitory activity of this compound was independent of the food matrix because no decrease in activity was observed in the bio-assay with *K. pneumoniae*.

Our results indicate that the selective agents applied in enrichment procedures will interfere with the recovery of *L. monocytogenes* from food and environmental samples. It has been shown previously that high numbers of competing flora will hamper the detection and identification of pathogens (Jung 1987, Beumer and Brinkman 1991).

The selective enrichment protocols for *Listeria* isolation formulated by the FDA for dairy products (Lovett et al. 1987) and by the USDA for meat (McClain and Lee 1988) are among the most frequently used. In the USDA method an acriflavine concentration of 12 mg l^{-1} in the primary enrichment broth (UVM1) and 25 mg l^{-1} in the secondary enrichment broth (UVM2) are applied. In the FDA procedure, a concentration of 15 mg l^{-1} is recommended in the *Listeria* enrichment broth (LEB). In several studies both methods have been compared for their ability to recover *Listeria* from food samples.

Table 4 Acriflavine (AF) induced inhibition zones (mm) of *Bacillus subtilis* (well assay) and nalidixic acid (NA) induced inhibition zones of *Klebsiella pneumoniae* by adding enrichment media (University of Vermont broth, UVM and Buffered *Listeria* Enrichment Broth, BLEB) and (1:9) suspensions of food in these media, directly after homogenization and after incubation of 24 h at 30°C, to wells in tryptone soya agar with 0.6% yeast extract added

Product		Inhibition zones (mm) of			
		B. subtilis (AF induced)		*K. pneumoniae* (NA induced)	
	pH at t=24h	t=0h[a]	t=24h	t=0h[a]	t=24h
UVM-broth	7.3	6	6	7	8
+ poultry meat	7.1	3	2	6	6
+ minced meat	7.1	2	1	7	7
+ pâté	7.0	2	2	6	6
BLEB	7.2	6	6	10	12
+ milk	6.8	2	2	11	9
+ brie	6.2	1	1	11	11
+ salad	6.0	3	1	10	10

[a] after homogenization

In studies with unstressed and stressed cells both methods recovered the pathogen equally well from ice cream (Hitchins 1989), seafood (Lovett et al. 1991), brie cheese and chicken rinse samples (Bailey et al. 1990b), blue and white mould cheese and

canned corned beef (Westöö and Peterz 1992). These results may be influenced by the rather high levels of *L. monocytogenes* (up to 5−6 cells ml^{-1}) and the low numbers of competitors in the artificially contaminated samples used.

Some studies, both with naturally and artificially contaminated samples, state a better performance using the FDA method. In a most probable number (MPN) procedure with naturally contaminated cheese (Brie) the MPN estimate using USDA broth ($9.3x10^3$ cfu g^{-1}) was more than one log unit lower than using the FDA broth (> $2.4\ x10^5$ cfu g^{-1}) or a direct count ($5.0x10^5$ cfu g^{-1}) on modified McBride Agar. From additional experiments it became clear that addition of glucose and phytone (a papaic digest of soy protein) to USDA broth improved its recovery efficiency, but not up to that of FDA broth (Swaminathan et al. 1988). Similar results were reported with seafoods spiked with heat-treated cells (Lovett et al. 1991). The greater selectivity of the USDA procedure was explained by the greater selectivity of the USDA isolation medium (i.e. lithium chloride phenylethanol moxalactam agar (LPM) (Lee and McClain 1986). However, the results presented in this study suggest that this may also be explained by the higher acriflavine concentration applied in the USDA method.

In experiments reported by Bailey et al. (1990a) only the USDA broth consistently allowed recovery of heat-injured *L. monocytogenes*. It was supposed that the presence of glucose and the lack of an adequate buffering system in LEB accounted for the lower efficacy. The lower pH in the FDA enrichment broth and, as a consequence of this, a reduced inhibitory activity of acriflavine (as shown in this study) resulting in a better growth of the competitive microflora, may have masked the presence of this pathogen. Similar results, although statistically not significant, were obtained in comparative studies (Van Netten et al. 1989, Warburton et al. 1992). The USDA procedure and the procedure described by Van Netten et al. (1989) detected *L. monocytogenes* in 74% of the samples known to contain this pathogen (n=121), only a slight difference from the 65% detected by the FDA protocol (Hayes et al. 1992). Based on the collective data (n=499) of three interlaboratory studies 199 (42%) and 210 (45%) samples were found positive by the modified FDA and USDA methods, respectively (Warburton et al. 1992). However, modifications in the original description of the FDA method considerably influenced the results obtained. An increase in the numbers of samples positive for *L. monocytogenes* (from 47%−87% of the 112 positive samples) was observed by incorporation of LPM, used in the USDA procedure, and Oxford medium (Curtis et al. 1989) as additional isolation media. A smaller increase (from 68%−86% of the 102

positive samples) was observed in the USDA procedure incorporating modified Mc Bride agar (MMA), used in the FDA method, and Oxford medium (Warburton et al. 1991a). In studies with a variety of food products LPM or Oxford medium have been proven to be more effective than MMA in recovering *L. monocytogenes* (Brackett and Beuchat 1989, Cassidy et al. 1989, Heisick et al. 1989a 1989b, Van Netten et al. 1989, Northolt 1989, Lund et al. 1991). Because the FDA enrichment broth does not appear to have a significant inhibitory effect on competing microorganisms, the use of media more selective than MMA will improve isolation results. Improved detection of *L. monocytogenes* by incorporating additional media would be expected to be less evident in protocols already employing an effective isolation medium such as LPM in the USDA method.

Another improvement of the FDA method was its change to a two-stage enrichment procedure. The use of Fraser broth (FB), a modification of UVM (Fraser and Sperber 1988), as secondary enrichment medium resulted in a score of 104 (92.9%) of the 112 positive samples. Unfortunately, FB was only applied in the FDA modification including additional isolation media. Therefore, its impact on the original procedure is not known (Warburton et al. 1991a). Previous studies have shown that two-stage enrichment procedures improve the detection of *Listeria* spp. in foods by inhibiting the growth of competitive microorganisms (Lee and McClain 1986, Lammerding and Doyle 1989, Fernandez-Garayzabal and Genigeorgis 1990). Because UVM and its modification FB maintain their pH closer to neutrality than LEB (Bailey et al. 1990a 1990b, Warburton et al. 1992), the moderately superior performance of the USDA method may be a result of increased inhibitory activity of acriflavine.

Recently, the International Organization for Standardization (ISO) published a draft international standard (DIS 11290−1) for the detection and enumeration of *L. monocytogenes* in food and animal feeding stuffs. This method is based on a two-stage enrichment procedure with isolation steps from the primary enrichment broth (half FB, acriflavine 12 mg l^{-1}) after one day of incubation and from the secondary enrichment broth (FB, acriflavine 24 mg l^{-1}) after two days of incubation on both Oxford and PALCAM agar (Anonymous 1995).

Previous comparative studies (Warburton et al. 1991a 1991b 1992) combined with this study indicate that the two-stage ISO procedure can be recommended for the detection of *L. monocytogenes*. Because of the large variety of food and feed products, this method may not be necessarily applicable to all products. Future studies with this

method should include an isolation step after 24 h incubation of the secondary enrichment broth, and pH measurements in the broth at the time of isolation to determine the effect on pH by the food sample.

Because in comparative studies no attention was paid to the ratio *L. monocytogenes* to other *Listeria* spp. information about the under-reporting of *L. monocytogenes* is not available. In our study it was shown that *L. monocytogenes* serotype 4B was most affected by acriflavine. This is in agreement with the fact that serotype 4B has been isolated far more frequently from listeriosis patients, than the serotypes isolated from incriminated food products (Farber and Peterkin 1991). For that reason it is worthwhile to introduce in protocols for the detection of *L. monocytogenes* an isolation medium which allows identification of *L. monocytogenes* in presence of high numbers of other listerias. Enhanced haemolysis agar (EHA), a medium described by Cox et al. (1991a 1991b) and modified by Beumer et al. (1992) is suitable for this, because *L. monocytogenes* can be distinguished from non-pathogenic listerias on basis of hemolysis. In procedures where rapid methods such as Elisa's or DNA probes are applied, there is a possibility that presence of *L. monocytogenes* will be masked by faster growth of other *Listeria* spp. Therefore, methods detecting the genus *Listeria* should be preferred. EHA may be used for the confirmation of presumptive positives.

It has been reported that the predominant microflora in enrichment media for *Listeria* spp. consists of lactobacilli, enterococci and Gram-negative microorganisms, including pseudomonads (Duffy et al. 1994). Conflicting results have been reported about the effect of these bacteria on *Listeria* growth. Lactobacilli and enterococci may produce compounds e.g. bacteriocins, some of which are active against listerias (Harris et al. 1989), whereas the effect of pseudomonads is both stimulating and inhibitory (Farrag and Marth 1989). Competition may be a result of the types of microorganisms, rather than the overall numbers present (Tran et al. 1990, Dallas et al. 1991, Duffy et al. 1994).

Although the proposed ISO-method for the detection of *L. monocytogenes* is an improvement in comparison with the FDA and USDA methods, it is obvious that modifications or supplementation of the enrichment media are still necessary.

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4

OPTIMIZATION OF ENHANCED HAEMOLYSIS AGAR (EHA), A SELECTIVE MEDIUM FOR THE ISOLATION OF *LISTERIA MONOCYTOGENES*

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SUMMARY

The presence of *Listeria monocytogenes* in enrichment media can be masked by faster growth of other *Listeria* spp. Therefore enhanced hemolysis agar (EHA) is a good alternative for other isolation media, because the presence of a few *L. monocytogenes* colonies can be detected between a majority of colonies of other listerias on basis of hemolysis. In this study the hemolysis reaction in EHA was optimized.

4

INTRODUCTION

Of the six accepted species of *Listeria*, *Listeria monocytogenes* and *L. innocua* occur most frequently in foods. *L. welshimeri* and *L. seeligeri* are less frequently found in foods, but may be isolated more often from environmental samples. *L. ivanovii* and *L. grayi/murrayi* are only rarely present in foods. During enrichment of samples, faster growth of *Listeria* spp. other than *L. monocytogenes* can mask the presence of this pathogen (Petran and Swanson 1993, McDonald and Sutherland 1994) (Chapter 3). All commercially available isolation media for *Listeria* suffer from the same disadvantage in that they offer no elective feature whereby *L. monocytogenes* can be distinguished from non-pathogenic listerias. This is an important factor in the routine analysis of foods. Even the selection of five suspect colonies at random from such media could lead to the detection only of non-pathogenic listerias, even though a few *L. monocytogenes* were present on the plate. The most important differentiating features for the listerias commonly found in foods and food environments will therefore be hemolysis, the CAMP reaction and xylose fermentation (Seeliger and Jones 1986). These characteristics were used to develop a medium for the isolation of *L. monocytogenes*. On this medium, enhanced haemolysis agar (EHA), described by Cox et al. (1991a 1991b), *L. monocytogenes* can be distinguished from non-pathogenic listerias on the basis of hemolysis.

Previously, a blood agar overlay technique has been described by Blanco et al. (1989), which relies on the hemolytic properties of *L. monocytogenes*. However, hydrolysis of esculin (1 g l^{-1} in base medium) could give rise to acid formation from the liberated glucose and lead to unreliable results, a fact not sufficiently discussed by these authors. The technique does not seem to be applicable to all selective media currently in use, since hemolysin, according to these workers, is not produced on all media. The medium requires 64 h incubation, with an overlay of blood agar at 48 h adding some inconvenience.

Development of enhanced haemolysis agar (EHA)

The production of grey-green/brown, black-haloed colonies with a dark, depressed centre on Oxford medium (Curtis et al. 1989a) is almost diagnostic for the genus *Listeria*. The blackening is caused by the hydrolysis of the glycoside esculin to glucose, and the aglycone esculetin, the latter forming an insoluble black complex with iron, present in the medium as ferric citrate. Only a few other organisms present in food enrichments can grow on Oxford medium, split esculin, and have the typical shape and

colour of *Listeria* colonies. For this reason, the Oxford medium was chosen as a starting point for development of a selective medium with improved diagnostic features.

The first diagnostic feature of the medium is based on the production of ß-glucosidase by *Listeria* spp. This feature is exploited in Oxford agar and some other media as al-ready mentioned, and is usually detected by the hydrolysis of esculin. To overcome the problem of acid formation from glucose, that might give lysis of blood cells and lead to spurious results, 4-methylumbelliferyl-ß-D-glucoside (MUßG) instead of esculin was ad-ded. It was decided to use this compound, since on hydrolysis it releases methylumbelliferone, which fluoresces in UV-light (366 nm) and is detectable at low levels in the me-dium. Esculin could be used at the same level and hydrolysis detected by loss of fluorescence. However, this is more inconvenient than looking for fluorescence. The use of the iron-esculetin reaction is not convenient, because it may mask the hemolysis zones at the level used (1 g l^{-1}). Lower levels of esculin and iron were investigated, but the blackening reaction was too weak in some cases, to be used as a differentiating factor.

Secondly, *Listeria* colonies have a morphology distinct from any other organisms that might grow on the medium (see 'description of colonies').

The third important differential feature is based on the ability of *L. monocytogenes* and some strains of *L. seeligeri* to lyse ovine erythrocytes, in what is known as the CAMP reaction (Christie et al. 1944, Munch-Petersen et al. 1945, Fraser 1962, Brzin and Seeliger 1974). This reaction is caused by the presence of a factor (CAMP factor), a 23kD polypeptide (Chakraborty and Goebel 1988) of which the exact nature is unknown, that is able to completely lyse ovine erythrocytes in the vicinity of a non- or weakly-hemolytic ß-hemolysin-producing *Staphylococcus aureus* culture, growing on blood agar. Compared to the normal hemolytic reaction of *L. monocytogenes*, the CAMP factor related hemolysis is enhanced; hence, EHA. The full basis for the CAMP reaction is not understood completely for all of the other organisms that show this reaction, including *L. monocytogenes*. However, it is known that the factor produ-ced by *St. aureus* is an enzyme, sphingomyelinase (sphingomyelin choline phosphohydrolase EC 3.1.4.12). This enzyme removes sphingomyelin from the erythrocyte membrane, but does not cause the cell to lyse (Cry et al. 1963, Bernheimer et al. 1979). The CAMP factor completes the lysis of the erythrocyte membrane. Ovine or ox erythrocytes are particularly rich in sphingomyelin, and are thus the best for demonstrating this reaction (De Gier and van Deenen 1961, Nelson 1967, Colley et al. 1973, Zwaal et al. 1973).

Description of colonies on EHA

In UV-light (366 nm) *Listeria* colonies are surrounded by a very diffuse zone of light, whitish-blue fluorescence after 48 h incubation. Close-up, the colonies are strongly fluorescent, bluish-white on the outer edges with a yellow, matt fluorescent centre. When viewed from 50 cm or more, they appear to be entirely whitish-blue and fluorescent. When colonies are small, the yellow centre may not be visible. The fluorescence can be seen forming at 16-24 h, but colonies are not usually large enough for manipulation at this stage.

In daylight, *Listeria* colonies are translucent, shiny, entire, slightly raised with a more prominent, whitish centre after 48 h incubation. They are whitish to light, buttery yellow in colour, due to uptake of acriflavine. Colony size is normally around 1.5-2.0 mm diameter when in the centre of the agar plate. They may be somewhat smaller at the edges of the plate or when surrounded by numerous other colonies.

When lit from below, *L. monocytogenes* colonies are surrounded by a distinct zone of hemolysis. *L. innocua* shows no hemolytic zone, but shows all other features noted above. *L. seeligeri* produces zones of hemolysis that are less prominent than those of *L. monocytogenes*. Unlike the observations of Curtis et al. (1989b), incubation at 37°C did not inhibit the growth of these strains. The strains of *L. ivanovii* tested, could not grow on the medium. It would be expected that *L. welshimeri, L. grayii/murayii* (CAMP-negative) would react in the same way as *L. innocua* on EHA.

Using this medium, we observed that hemolysis of *L. monocytogenes* was variable. In literature it has been reported, that various agents may affect hemolysis (McFaddin 1980, Cowart 1987, Farber and Peterkin 1991, Fernandez-Garayzabal et al. 1992). Therefore, the aim of this study was to improve the hemolytic reaction in EHA described by Cox et al. (1991a, 1991b), and to compare the new formula in a collaborative study.

MATERIALS AND METHODS

Listeria strains

L. monocytogenes 1 (1/2b), 106 (1/2a), 144 (4b), 285 (unknown), 294 (1/2a), 311 (3a), A (unknown), An10A (1/2a), L4492 (4b); *L. innocua* (#=5); *L. grayi* (#=4); *L. welshimeri* (#=3); *L. seeligeri* (#=3) and *L. ivanovii* (#=2) were used. These strains were all isolated from food or food environments.

Strains were stored at -20 °C in brain heart infusion broth (BHIB, Difco 0037-01-6) + 20% glycerol. Test suspensions of *Listeria* spp. were prepared by diluting overnight (16-20 h, 37 °C) cultures in BHIB with physiological saline solution (NaCl 8.5 g l^{-1}) to a concentration of about 500 cfu ml^{-1}.

Enhanced haemolysis agar (EHA)

Composition of the original medium (Cox et al. 1991a 1991b) (amount l^{-1}): Columbia blood agar base (CAB, Oxoid CM 331) 39 g, 4-methylumbelliferyl-β-D-glucoside (MUβG, Sigma M3633) 50 mg, lithium chloride 10 g, Oxford supplement (Oxoid SR 140) 2 vials, sphingomyelinase (Sigma S8633) 10 units, sheep blood (sterile defibrinated) 50 ml. Because the Oxford supplement, used in EHA, is less effective in suppressing competive microorganisms (Gunasinghe et al. 1994), it was replaced by the PALCAM supplement (Merck 12122).

CAB and MUβG were weighed in distilled water (950 ml), after soaking the pH was adjusted to 7.0 ± 0.2 and the medium was sterilized for 15 min at 121 °C. After cooling the medium to 50 °C the other ingredients were added in the following order. Lithium chloride was added first as a 50% (w/v) solution in distilled water, then the PALCAM supplement dissolved in 1 ml of distilled water, followed by the sphingomyelinase dissolved in 1 ml phosphate buffered saline [PBS, Composition (amount l^{-1}): $Na_2HPO_4.2H_2O$ 1.44 g; NaCl 8 g; KCl 0.2 g]. Finally, the sheep blood was added, the medium was shaken carefully and thin plates (10 ml) were poured using a sterile pipet. In EHA-MM the basal medium CAB was replaced by agar noble (Difco 0142-01, 10 g l^{-1}) and 270 ml of a stock solution of minimal medium (MM) (Beumer et al. 1994) (Chapter 10) to study the influence of several components on the hemolysis of *L. monocytogenes* in EHA. Agar noble and MUβG were dissolved in distilled water (670 ml). After sterilization and subsequent cooling of the medium to 50-55 °C the stock solution of MM (270 ml, heated in a waterbath to approximately 45 °C) was added first, followed by the other ingredients mentioned above.

Reagents tested for improved or impeded hemolysis in EHA-MM and EHA

Acriflavine (Fluka 01675), $CaCl_2.2H_2O$ (Merck 102381), ceftazidime (Glaxo), cholesterol (Acros Chimica 11.019.58), $CoCl_2$ (Merck 802540), cysteine (Merck 102838), EDTA (Merck Titriplex III 108418), Fe(III)-citrate (Merck 103862), $FeCl_2.4H_2O$ (Merck 103860), K-tellurite (Merck 5464), $LiCl_2$ (Merck 105675), $MgCl_2.6H_2O$ (Merck

105832), tri-Na-citrate.$2H_2O$ (Merck 106448), PALCAM supplement (Merck 12122), polymyxin-B-SO_4 (Pfizer) and polyclonal antibodies against *Listeria* (Kirkegaard and Perry 01-90-90). All the reagents to be tested for their effect on hemolysis were added as filter sterilized solutions to 100 ml EHA-MM. Plates were poured and inoculated with test suspensions (0.1 ml) of *Listeria*. After incubation for 48 h at 37 °C growth and hemolysis were compared with the performance on the EHA medium without added reagents.

Recovery of *L. monocytogenes* in a collaborative study using reference samples

Sixteen laboratories compared counts of *L. monocytogenes* in reference samples (In 't Veld and De Boer 1991) using EHA. Tryptone soya agar EH (TSA-EH, Difco 0028-17-9) was used as agar base and the concentration LiCl was 5 g l^{-1}. EHA plates prepared in one batch as described above were distributed to the participants. PALCAM agar was prepared by each participating laboratory (Merck, 11755). Each laboratory received a series of 10 test capsules (ca 5.10^3 cfu). Counts were performed as described previously (In 't Veld and De Boer 1991). Typical colonies were inoculated on TSA plates and incubated for 24 h at 37 °C. With single colonies the following tests were performed: Gram reaction (Gram-positive bacilli or coccobacilli), motility (umbrella type), presence of catalase (+) and oxidase (-) (Lovett 1988) and hemolytic activity (Dominquez et al. 1986).

RESULTS AND DISCUSSION

All *Listeria* strains tested showed fluorescence under UV-light (366 nm) due to the splitting of MUβG on EHA-MM. In addition, *L. monocytogenes*, *L. ivanovii* and *L. seeligeri* strains showed hemolysis. *L. seeligeri* produced zones of hemolysis that were less prominent than those of *L. monocytogenes* and the strains of *L. ivanovii* tested showed hardly any hemolysis. Both species are not frequently isolated from food or food environments and can easily be distinguished from *L. monocytogenes* by a biochemical identification (Cox 1991a). Zones of hemolysis were strain dependent, however, no significant differences were observed between the strains tested. In Table 1 the effects of added agents on hemolysis of two strains in EHA-MM are shown. Addition of cobalt, cysteine, magnesium and potassium tellurite should improve hemolysis (McFaddin 1980, Farber and Peterkin 1991, Fernandez-Garayzabal 1992). In our experiments, this was only demonstrated for cysteine and magnesium.

Table 1 Effect of various agents on hemolysis of *Listeria monocytogenes* (strain A and strain L4492) in enhanced hemolysis agar, based on a minimal medium (EHA-MM)

Added agent	Concentration		*L.m.* A	*L.m.* L4492	Supposed effect (reference)
$CoCl_2$	2, 20	mM	−−	−−	+ Mc Faddin (1980)
Cysteine	2	mM	++	=	+ Farber and Peterkin (1991)
	4	mM	+	+	
K-tellurite	0.16	mM	−−	−−	+ Fernandez-Garayzabal (1992)
$MgCl_2$	10	mM	+	+	+ McFaddin (1980)
	30, 70	mM	++	++	
	100	mM	−	−	
$CaCl_2$	10	mM	+	=	− McFaddin (1980)
	50, 100	mM	−	−	
	100	mM	−−	−−	
Cholesterol	0.01	mg ml^{-1}	+	++	− Farber and Peterkin (1991)
	0.05	mg ml^{-1}	=	=	
	0.1	mg ml^{-1}	+	+	
$FeCl_2$	0.01	mM	−	−	− Cowart (1987)
	1.0	mM	+	++	
	10, 100	mM	−	−	
Fe(III)citrate	0.01, 0.1	mM	+	++	− McFaddin (1980)
	0.3	mM	+	=	
	0.9	mM	++	+	
Na-citrate	1	mM	=	=	− McFaddin (1980)
	10	mM	+	+	
EDTA	1, 4	mM	−−	−−	− McFaddin (1980)
Antibodies	0.5, 1	μg ml^{-1}	+	+	
	2	μg ml^{-1}	−	=	

= hardly any effect on hemolysis; − poor hemolysis; −− no hemolysis
+, ++ increased hemolysis (+ up to 25%; ++ 25-50%)

Addition of calcium, cholesterol, iron, citrate and EDTA should inhibit hemolytic activity (McFaddin 1980, Cowart 1987, Farber and Peterkin 1991). However, for cholesterol and citrate this effect was not observed. The reason that effects of some agents on hemolysis in our experiments did not agree with data published earlier, might be due to the use of a minimal medium, by which interference with components of complex culture media is out of the question.

Both LiCl and the PALCAM supplement negatively affect hemolysis. In the PALCAM supplement acriflavine and polymyxine-B-SO_4 had the greatest effect. Synergistic effects between LiCl and the PALCAM supplement, nor between acriflavine and polymyxine-B-SO_4 were observed (data not shown). Because of the widespread use of PALCAM supplement in food microbiology and the restricted effects on hemolysis, we did not change the formula of this supplement. Instead of this, we performed several experiments to investigate if it was possible to reduce the LiCl concentration in the medium. Using half the normal concentration of LiCl (5 g l^{-1}) improved hemolysis and resulted in slightly higher counts, but differences were not statistically significant. This agreed with the findings of Poysky et al. (1993).

A lower concentration of LiCl may also favour the growth of competitive microorganisms. For that reason 0.2 ml of dilutions (1:4) of 50 food products (pâté, vegetable salads, cheese) were streaked on the surface of EHA both with 5 and 10 g l^{-1} LiCl. Great differences in numbers of colonies or colony size between the two concentrations could not be demonstrated. Only hemolytic colonies of *Bacillus* spp. sometimes disturbed reading the hemolysis reaction of *L. monocytogenes*. By adding nalidixic acid (40 mg l^{-1}) to EHA this problem could be overcome (data not shown).

It will be clear from the results presented in Table 1 that, due to differences in chemical composition (Bridson 1990), (partial) substitution of CAB by peptones or agar bases in EHA can influence the hemolysis. Substitution of peptone in CAB by Bactopeptone (Oxoid L34), Tryptone (Oxoid L42), Tryptone T (Oxoid L43), Soya peptone (Oxoid L44) or Tryptose (Oxoid L47) resulted in all cases in a (slightly) impeded hemolysis. No hemolysis at all was observed when CAB was substituted by TSA (Biokar BK047). The use of TSA (Oxoid CM 131) slightly improved hemolysis, however, best results for hemolysis were obtained with TSA-EH (Difco) (data not shown). There are no data available about the chemical composition of TSA-EH. However, it will be clear that the concentration of factors that interfere with hemolysis will be lower compared with the other basal media tested. Since its considerable effect

on hemolysis its use in blood containing media can be recommended. In further experiments CAB was substituted by TSA-EH.

In the original formula of EHA, sheep blood is used in a concentration of 5%. This amount is optimal, because lowering the concentration (down to 2%) did not improve hemolysis, whereas increasing the concentration up to 10% resulted in no hemolysis. This might be due to the chemical composition of the blood plasma (e.g. cholesterol, antibodies against *Listeria*), because simultaneous addition of increasing concentrations

Table 2 Mean counts and standard deviation obtained with reference samples of *Listeria monocytogenes* on enhanced hemolysis agar (EHA) and PALCAM agar after 48 h incubation at 37°C

Laboratory	Medium compared with EHA	Mean counts and standard deviation on Own medium	EHA
1	PALCAM Merck 11755	49.9 ± 10.3	26.8 ± 6.3
6	PALCAM Merck 11755	32.8 ± 5.3	32.8 ± 6.4
7	PALCAM Merck 11755	29.7 ± 11.8	32.7 ± 12.6
13	PALCAM Merck 11755	37.0 ± 5.1	37.9 ± 4.3
14	PALCAM Merck 11755	43.3 ± 9.2	49.8 ± 6.4
	Mean	**38.5 ± 11.4**	**34.5 ± 10.2**
2	PALCAM Merck 11755, yolk	32.2 ± 8.8	37.2 ± 7
4	PALCAM Merck 11755, yolk	61.1 ± 10	42.8 ± 6.8
5	PALCAM Merck 11755, yolk	41.7 ± 8	41.9 ± 7.5
9	PALCAM Merck 11755, yolk	54.7 ± 8.9	46.0 ± 6.8
11	PALCAM Merck 11755, yolk	32.7 ± 10.1	42.8 ± 7.9
12	PALCAM Merck 11755, yolk	50.0 ± 9.2	46.3 ± 9.6
15	PALCAM Merck 11755, yolk	49.3 ± 8.1	49.0 ± 9.8
	Mean	**45.9 ± 13.6**	**42.2 ± 8.3**
8	PALCAM Oxoid CM 877	44.9 ± 6.5	35.8 ± 8.2
16	PALCAM Oxoid CM 877, yolk	43.5 ± 7.7	49.0 ± 9.8
	Mean	**44.2 ± 7.1**	**42.4 ± 11.2**
3	Oxford Agar CM 856	38.1 ± 6.5	36.2 ± 7.6
10	Oxford Agar CM 856	37.1 ± 5.8	34.1 ± 9.6
	Mean	**37.8 ± 6.0**	**35.2 ± 8.7**
	Mean (all participants)	**42.4 ± 12.1**	**39.0 ± 10**

(up to 10%) of blood plasma (sheep) to EHA (with 5% sheep blood), completely inhibited hemolysis. With blood from horse and guinea-pig hardly any hemolysis was observed. Only slight differences in hemolysis were found by using blood from duck, hen, man, pigeon and rabbit. However, when blood from individual animals was used, zones of hemolysis (mm) varied up to 50% (data not shown).

The results of a collaborative study with EHA are presented in Table 2. Sixteen laboratories compared counts of *L. monocytogenes* in reference samples using PALCAM or Oxford Agar and EHA. The mean counts on PALCAM and Oxford Agar were somewhat higher than those on EHA, but no significant difference was found. Higher mean counts were found by laboratories which added egg yolk to PALCAM. Since the mean EHA counts from these laboratories were also higher, this may be contributed to inter-laboratory effects, rather than the higher productivity of PALCAM media with egg yolk added.

Independent of our study, modifications (both for products with low and high numbers of competitors) of EHA described by Cox et al (1991a 1991b) were proposed for the enumeration of *Listeria* spp. in foods (Heisick et al. 1995). Though these media were tested with a limited number of samples, results were promising. Occasionally, with low-level inocula, *Listeria* was not detected.

It is also possible to use other markers (phospholipase C production in egg yolk containing media) to differentiate *L. monocytogenes* from other listerias (Coffey et al. 1996). However, this principle was only investigated using pure cultures, whereas the utility of EHA already has been proven in an evaluation of confirmation and identification methods (Beumer et al. 1996a) (Chapter 6) and in a study concerning the occurrence of *Listeria* spp. in domestic environments (Beumer et al. 1996b) (Chapter 11).

In conclusion, we found that EHA based on TSA-EH (Difco), with LiCl (5 g l^{-1}) is a good alternative for PALCAM agar and other isolation media, since *L. monocytogenes* can be differentiated from other listerias on basis of hemolysis. The final composition of the medium (amount l^{-1}) is: trypticase soy blood agar base EH (TSA-EH, Difco 0028-17-9) 40 g; 4-methylumberiferyl-β-D-glucoside (MUβG, Sigma M3633) 50 mg; lithiumchloride 5 g; PALCAM supplement (Merck 12122) 2 vials; sphingomyelinase (Sigma S8633) 10 units; sheep blood (sterile defibrinated) 50 ml. The use of EHA as direct plating- or isolation medium is to prefer to the use of media applying an upper layer of blood agar, because this doubles the incubation period. Further (comparative) studies with this medium in direct plating and/or enrichment procedures should confirm this.

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5

A COMPARISON OF RAPID METHODS FOR THE DETECTION OF *LISTERIA* SPP. AND *LISTERIA MONOCYTOGENES*

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This chapter has been submitted

SUMMARY

In this study it is shown that there are some *Listeria* and *Listeria monocytogenes* rapid test kits on the market which are superior to the standard USDA method. Considerable differences exist among various test kits, which may be contributed to the enrichment protocols, the detection limits of the tests and the concentration of *Listeria* cells in the samples investigated.

Samples with high numbers of *Listeria* are readily detected by most rapid methods. Consequently, these samples can be considered as 'positive controls'. The results obtained with these samples should not be included in the evaluation of the method. However, the performance on samples with low numbers of this pathogen varied from 6% to 94% of samples detected as positive. In collaborative and comparative studies, rapid methods are sometimes compared with standard protocols using completely different enrichment protocols. The use of identical protocols is recommended. In those cases where this is not feasible, cross-checking of the enrichment media is necessary.

5

INTRODUCTION

Members of the genus *Listeria* are ubiquitous in nature. Of all *Listeria* species only *Listeria monocytogenes* has regularly been implicated in human and animal disease. Epidemics of foodborne listeriosis have raised concern about the incidence of *L. monocytogenes* in foods. Because of the psychrotrophic nature of this microorganism, its growth on ready-to-eat contaminated food products during refrigerated storage could increase the risk of listeriosis. Thus, it is important to emphasize that processors can not rely entirely on storage at refrigeration temperatures to control this pathogen (Cox et al. 1989).

As the food laws of many countries insist on the absence of *L. monocytogenes* (which means not detectable in 25 g of food product), enrichment procedures are necessary for the successful detection of this bacterium. There is some confusion about the best method for isolating this organism. This is partly due to the fact that two methods exist, depending on the type of food. The US Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS) has developed methods primarily intended for meat and poultry products (McClain and Lee 1988). The US Food and Drug Administration (FDA) has formulated methods for analysis of dairy products and fish and shell-fish (Lovett et al. 1987). Some Western European countries propose for certain food products absence in 0.01 g (<100 cfu g^{-1}) which allows the use of direct plating methods.

Since the present methods for the recovery and identification of this pathogen in food are both time consuming and labour intensive, rapid and/or automated detection methods have been developed. Food producers and distributors as well as public health authorities have a great interest in rapid methods. In principle, these methods permit a more efficient control of raw materials, processes and products and they may play an important role in food trade and product liability. That is why it is important that test results obtained with newly developed rapid methods are reliable and will be accepted by all parties involved. General acceptance of a new method will only be realized after extensive testing and validation has shown that the method is sufficiently sensitive, specific, user friendly and not too expensive.

In scientific literature new methods are described at a very high rate. Particularly methods based on in-vitro DNA amplification, applying the polymerase chain reaction (PCR) are being developed (Hofstra et al. 1994), but an alternative, in which RNA is being amplified (Uyttendale et al. 1995) has also been proposed. Although these

methods are very promising, none of them has appeared on the market as a ready-for-use test kit with complete and detailed directions for use. For the time being interference of the food matrix in the performance of nucleic acid amplification reactions remains a major hurdle to be taken before a successful introduction of these methods in routine microbiological analysis of food can be expected (Lantz et al. 1994).

The aim of this work was to study the performance of a number of commercially available test kits for the detection of *Listeria* spp., using naturally contaminated food samples.

MATERIAL AND METHODS

Rapid methods

Enzyme-linked immunosorbent assays (ELISA's): *Listeria* Immunoenzymatic Detection Kit (Transia, France), *Listeria*-Tek (Organon-Teknika, Belgium), *Listeria* Visual Immunoassay (Tecra, Australia), an enzyme-linked fluorescent immunoassay (ELFA): Vidas-Lis (bioMérieux, France) and an immunological test in dipstick format: *Listeria* Rapid Test (Unipath/Oxoid, UK) were used for the detection of *Listeria* spp. For the specific detection of *L. monocytogenes* an ELFA: Vidas-LMO (bioMérieux, France) and a DNA-probe: *Listeria monocytogenes* assay (Gene-Trak, USA) were used.

Procedure

All samples used in this study were bought at retail outlets and markets, transported in melting ice, in insulated cool boxes to the laboratory, and investigated the same day using the procedures formulated by the Food and Drug Administration (FDA) for dairy products, vegetables and environmental samples (Lovett et al. 1987) and the US Department of Agriculture (USDA) method for meat (McClain and Lee 1988) and the rapid methods listed above according to the manufacturers instructions. Test samples were prepared by thoroughly mixing about 100 g of the samples. Twenty-five grams of the test sample were added to the primary enrichment broths (225 ml) and homogenized for 1 min with a stomacher. All rapid tests were performed after a two-stage enrichment procedure in liquid media, with the exception of the DNA-probe, which employs a solid medium for the secondary enrichment. Moreover, 3x0.33 ml from the primary enrichment broths were transferred on the surface of three enhanced hemolysis agar plates (EHA, composition and preparation as in chapter 4) directly after stomacher treatment. Plates were incubated for 48 h at 37 °C. Typical *Listeria* colonies (fluorescent under

UV light (366 nm) and in case of *L. monocytogenes* showing hemolysis), were purified by streaking on tryptone soya agar (TSA Oxoid CM 131) and incubated for 1-2 days at 30 °C. Colonies demonstrating a characteristic blue colour under Henry illumination were tested for Gram reaction (Gram-positive bacilli or coccobacilli), presence of catalase (+) and oxidase (-) and further identified using API *Listeria* (bioMérieux, France). For each *Listeria* sp. a reference strain was included (Beumer et al. 1996b) (Chapter 6).

The performance of the proposed isolation media in the enrichment protocols: lithium chloride phenylethanol agar (LPM) and PALCAM agar, was compared with that of EHA. After incubation for 2 d at 30 °C (EHA 37 °C) growth (performance) of *Listeria* spp. and competitive microflora on isolation media was valuated from 0 (no growth) to 4 (growth on entire plate). Suitability of the media for the isolation of *Listeria* spp. was indicated with the performance value (PV). This value ($0 < PV < 4$) is the quotient of the PV of *Listeria* spp. and the PV of competititors. Typical colonies from the isolation media were purified on TSA and further identified as described above.

At all stages where rapid tests and/or isolation media were applied, pH measurements were performed.

Detection limit

The detection limits of the rapid tests were determined by adding to the secondary enrichment broths of meat products known to be free of *Listeria* spp., known numbers (10^5-10^7 cfu ml^{-1}, with increments of 0.25 log unit) of overnight cultures (20-22 h at 30°C) of *Listeria* spp. (*L. innocua*: 3 strains and *L. monocytogenes*: 2 strains) isolated from food or food environments. The detection limit for the Gene-Trak *Listeria* assay was determined in a different way. The growth on lithium chloride ceftazidime agar (LCA) was swabbed off and suspended in 1 ml of phosphate buffered saline (PBS). Known numbers of *L. monocytogenes* (3 strains) were added to this suspension as descibed above for secondary enrichment broths.

RESULTS

Seven commercially available *Listeria* detection kits were compared with the conventional FDA and USDA methods for the detection of *Listeria* and/or *L. monocytogenes* in various series of samples of food products. In a total of 156 samples of meat products, presence of *Listeria* was demonstrated in 96 (62%) of the samples. In 37 (39%) of the

96 positive samples presence of *Listeria* could be demonstrated by direct plating on EHA, indicating that numbers in these samples were greater than 10 cfu g^{-1}. In 50 (32%) of the samples *L. monocytogenes* was detected. Percentages were lower for the other products investigated: cheeses 16% and 11%, vegetable salads 5% and 2% and for environmental samples 17% and 7% respectively. Because of the low number of samples containing *Listeria*, only results for meat products are shown (Tables 1a-1c).

In the first series (Table 1a), *Listeria* was detected in 27 (59%) of 46 samples by at least one of the methods used. With the traditional method presence of *Listeria* was

Table 1a Numbers of naturally contaminated samples of meat products (n=46), in which *Listeria* was detected using a traditional method (USDA), rapid methods and a direct plating procedure with enhanced hemolysis agar (EHA)

Isolated *Listeria* spp.	#[a]	USDA	Oxoid	Vidas-LMO[b]	EHA *L/Lmc*
innocua	12	10	7		5/-[d]
monocytogenes	6	5	4	5	-/3
monocytogenes/innocua	7	1			1/4
monocytogenes		2	5	4	
innocua		4	2	3	
monocytogenes+welshimeri	1				
monocytogenes				1	
welshimeri		1			
innocua/welshimeri	1	1			
welshimeri			1		
Total pos. for *Listeria* spp.	27	24	19		13
Percentage		89	70		48
Total pos. for *L. monocytogenes*	14	8	9	13	7
Percentage		57	64	93	50

[a] #= number of positive samples, [b] LMO= *Listeria monocytogenes*, [c] number of samples in which presence of *Listeria* (*L*) or *L. monocytogenes* (*Lm*) was shown by direct plating, [d] - = below the detection limit (<10 cfu g^{-1})

demonstrated in 24 (89%) samples, whereas the Oxoid Clearview test detected only 19 (70%) of the positive samples. *L. monocytogenes* was present in 14 samples (30%). The USDA method and the Vidas-LMO test detected this pathogen in 8 (57%) and 13 (93%) of these samples, respectively. With the Oxoid Clearview test, 9 (64%) of the samples positive for *L. monocytogenes* were detected. With direct plating on EHA presence of *Listeria* was demonstrated in 13 (48%) samples, and in 7 (50%) of these samples *L. monocytogenes* was detected.

In 31 (91%) of the 34 samples examined in the second series (Table 1b), *Listeria* was detected. A considerable difference was found in the yield of positive samples with the two ELISA's. Best results were obtained with the Tecra test: 30 (97%) of the 31 positive samples were detected. With the USDA method presence of *Listeria* was shown in 21 (68%) of the positive samples. The Transia test detected *Listeria* in only 10 (32%) of the positive samples. *L. monocytogenes* was present in 23 (68%) samples. The USDA method detected this pathogen in 17 (74%) of these samples. This is comparable with the results obtained with the Tecra test, which detected 18 (78%) of these samples. The Transia test detected *L. monocytogenes* in only 9 (39%) samples. Presence of *Listeria* was demonstrated by direct plating on EHA in 13 (42%) of the samples. *L. monocytogenes* was detected in this way in 8 (35%) of the samples positive for this pathogen.

In the third series *Listeria* was detected in 38 (50%) and *L. monocytogenes* in 13 (17%) of the samples tested (Table 1c). The Vidas test and the USDA method performed equally well and presence of *Listeria* was shown in 26 (68%) of the positive samples. With the *Listeria*-Tek (OT) only 13 (34%) of the positive samples were detected. All 13 samples in which presence of *L. monocytogenes* was demonstrated by isolation of this microorganism, were detected by the Gene-Trak DNA-Probe. With the traditional method only 7 (54%) of these samples could be detected. In six other samples the DNA-probe gave a positive signal. By repeating the enrichment step and subsequently streaking on the isolation media, it was possible to detect *L. monocytogenes* in three of these samples.

In 11 (29%) samples presence of *Listeria* was demonstrated by direct plating on EHA. *L. monocytogenes* was detected in this way in 4 (31%) of the samples positive for this pathogen.

Using the normal procedure (without repeated transfer) in 72 (75%) of the *Listeria*-positive samples, only one *Listeria* sp. was detected. In 23 (24%) samples two, and in one sample (1%) three different *Listeria* spp. were detected. In 16 (17%) of the samples

both *L. innocua* and *L. monocytogenes* were present. With the traditional method 71 (74%) and with the best performing ELISA from each series of samples (series 1, Oxoid Clearview; series 2, Tecra; series 3, Vidas-Lis) 75 (78%) of the 96 positive samples were detected. It is obvious that none of the methods detects all samples which could be confirmed with any of the methods applied to a given subset of samples. The USDA method detected 68 to 89% of these positively confirmed samples. The yields of positive samples of the various ELISA's varied from 32-97%.

The pH-values after 24 h incubation in the primary enrichment broths are presented in Table 2. UVM-1, half Fraser and modified Fraser broth all use the same strength of buffer ($Na_2HPO_4.2H_2O$ 12 g l^{-1}; KH_2PO_4 1.35 g l^{-1}). After incubation with the food product, the pH-values reached were not too low to damage *Listeria*. Buffered *Listeria* enrichment broth (BLEB) uses the same buffer and additional (K_2HPO_4 2.5 g l^{-1}), and

Table 1b Numbers of naturally contaminated samples of meat products (n=34), in which *Listeria* was detected using a traditional method (USDA), rapid methods and a direct plating procedure with EHA

Isolated *Listeria* spp.	#[a]	USDA	Tecra	Transia	EHA *L*/*Lm*[b]
innocua	4	1	4		2/-[c]
monocytogenes	18	14	18	6	-/5
monocytogenes/innocua	5				1/3
monocytogenes		3		3	
innocua		1	5	1	
seeligeri	2	1	1		1/-
welshimeri	2	1	2		1/-
Total pos. for *Listeria* spp.	31	21	30	10	13
Percentage		68	97	32	42
Total pos. for *L. monocytogenes*	23	17	18	9	8
Percentage		74	78	39	35

[a] # = number of positive samples, *b* number of samples in which presence of *Listeria* (*L*) or *L. monocytogenes* (*Lm*) was shown by direct plating, [c] - = below the detection limit (< 10 cfu g^{-1})

Table 1c Numbers of naturally contaminated samples of meat products (n=76), in which *Listeria* was detected using a traditional method (USDA), rapid methods and a direct plating procedure with EHA

Isolated *Listeria* spp.	#[a]	USDA	OT[b]	Vidas *Listeria*	Gene-Trak	EHA *L/Lm*[c]
innocua	20	14	4	8		4/-[d]
monocytogenes	5(+1)	3	2	3	5(+1)[e]	-/2
monocytogenes/innocua	4(+1)					1/2
monocytogenes		1	2	2	4(+1)	
innocua		1	2	2		
monocytogenes/grayi	2(+1)	1				1/-
monocytogenes				1	2(+1)	
grayi			1	1		
monocytogenes/welshimeri	1	1				
monocytogenes				1	1(+1)	
welshimeri				1		
mono./innocua/welsh.	1	1				1/-
monocytogenes				1	1(+2)	
innocua			1			
welshimeri				2		
innocua/grayi	1					
innocua		1				
grayi				1		
grayi	1	1		1		
welshimeri	2	1	1	1		
grayi/welshimeri	1					
grayi				1		
welshimeri		1				
Total pos. for *Listeria* spp.	38	26	13	26		11
Percentage		68	34	68		29
Total pos. for *L. mon.*	13(+3)	7	4	8	13(+6)	4
Percentage		54	31	62	100	31

[a] # = number of positive samples, [b] OT= Organon Teknika, [c] number of samples in which presence of *Listeria* (*L*) or *L. monocytogenes* (*Lm*) was shown by direct plating, [d] below the detection limit (<10 cfug^{-1}), [e] (+) confirmed via EHA after repeated transfer in Fraser broth

Table 2 pH-values, after incubation, in various enrichment broths recommended for the detection of *Listeria* spp.

Product	# Samples	LEB	UVM-1	½-FB	m-FB
Meat products	156	not done	6.4 ± 0.5	6.4 ± 0.2	6.5 ± 0.2
Cheese	55	5.7 ± 0.5	6.4 ± 0.3	6.5 ± 0.4	6.6 ± 0.3
Vegetable salads	55	5.4 ± 0.9	5.9 ± 0.3	6.1 ± 0.4	6.0 ± 0.3
Environmental samples	30	5.9 ± 0.4	6.2 ± 0.3	6.3 ± 0.3	6.3 ± 0.3

yielded similar pH-values as those obtained in UVM and Fraser broth (data not shown). Buffering capacity of LEB was poor (K_2HPO_4 2.5 g l^{-1}), especially when used for samples with fermentable carbohydrates.

In Table 3 the detection limits for the various rapid methods used in this study are listed. For most tests these were on the same level (Log cfu ml^{-1} 5.2−5.8), only the Transia test and the DNA probe required considerable more cells for a positive reaction.

Table 3 Detection limits of rapid methods for the detection of *Listeria* and *Listeria monocytogenes*

Test	*Listeria* concentrations (log cfu per ml)[a]
Gene-Trak[b]	6.8 ± 0.3
Transia	6.5 ± 0.3
Tecra	5.7 ± 0.3
Vidas	5.8 ± 0.3
Organon Teknika	5.5 ± 0.2
Oxoid	5.2 ± 0.2
Vidas-LMO[b]	5.2 ± 0.2

[a] cfu= colony forming units, [b] detects specifically *L. monocytogenes*

In Table 4 performance values (PV) are listed for the isolation media used. Low PV's are due to scarce growth of *Listeria* and/or abundant growth of the competitive microflora. The PV's for PALCAM and EHA were similar, whereas that of LPM was substantially lower.

Table 4 Performance values (PV)[a] of media used for the isolation of *Listeria* from enrichment media

Investigated products	#	PALCAM	EHA	LPM
Meat products	63	2.3	2.1	0.6
Cheese, Vegetable salads and Environmental samples,	72	2.3	2.2	1.2

[a] PV = growth of *Listeria* spp./growth of competitive microflora

DISCUSSION

For the detection of pathogens in food the availability of rapid tests is desirable and various commercial procedures, employing ELISA's or DNA-probes, can be applied. In this study the performance of seven of these tests was evaluated.

The traditional method detected 71 (74%) of the 96 samples in which presence of *Listeria* and/or *L. monocytogenes* was demonstrated. This is comparable with the total amount of 75 (78%) positive readings obtained with the best performing ELISA from each serie. However, a considerable variation in the yield of the ELISA's (32-97%) was observed. Transia had the lowest yield (32%), due to the detection limit, being the highest of all ELISA's (Table 3). In addition the composition of the media and the incubation temperature may have influenced the final level of listerias present in the enrichment media. Both enrichment media used in this test (UVM1 and Fraser broth) have a good buffering capacity. Only the rather low incubation temperature (26°C) may have affected results. So far no collaborative studies with this ELISA have been published.

By direct plating on EHA it was shown that in 37 (39%) of the 96 positive samples *Listeria* spp. were present in numbers >10 cfu g^{-1}. The performance (the percentage of positive samples detected) of the USDA method in these samples varied from 91-100%, for the ELISA's this was 100%, with the exception of the Transia test which scored only 69%. In the positive samples with low numbers of *Listeria* spp. ($<$ 10 cfu g^{-1}) the USDA method detected 50-79% of the positive samples, for the ELISA's the percen-

tages were (in descending order) Tecra (94%), Vidas (56%), Oxoid (43%) and Transia (6%). This confirms that the Transia test needs high numbers of cells in the enrichment broth for a positive signal.

The Tecra test detected 30 (97%) of the positive samples in the second series. The detection limit of this test is similar to those of both Vidas *Listeria* and the *Listeria*-Tek, and only slightly above that of the Oxoid Clearview test. Because all three tests use good buffered enrichment media, the excellent result of the Tecra test can be mainly attributed to the given subset of samples. It is likely that replacement by any of the two tests, would also have resulted in a high score for this series. Unfortunately, comparative studies with the recently developed Oxoid Clearview *Listeria* test, and the Vidas *Listeria* and Vidas-LMO tests have not been reported in literature. However, comparative results are available for the *Listeria*-Tek and the Tecra test (Noah et al. 1991, Nørrung et al. 1991). In one of these studies the efficiency of the *Listeria*-Tek and the Tecra Unique *Listeria* test were compared with the FDA culture method. Differences in results of the ELISA's and the traditional method were not statistically significant. However, differences between results of the two immunological methods were significant and could be attributed to the superiority of Fraser broth, used in the *Listeria*-Tek, over UVM-2, used in the Tecra test (Noah et al. 1991). The poorer performance of UVM-2 was most likely due to the high concentration of acriflavine present in this medium. Recently, it has been demonstrated that this inhibitor, commonly used in selective media for *Listeria* spp. affects particularly the growth of *L. monocytogenes* (Beumer et al., 1996a) (Chapter 3). In another study it was concluded that the application of the *Listeria*-Tek test is limited for the detection of *Listeria* spp. in raw food products where the number of *Listeria* is usually low and the levels of the competitive microorganisms are high (Nørrung et al. 1991). In samples where the *L. monocytogenes* count was above 3 cfu g^{-1}, the ELISA and the USDA procedure proved to be equally sensitive. Only the development of efficient enrichment procedures, which favour growth of *Listeria* spp. will assure the successful application of rapid methods with detection limits of about 10^5-10^6 cfu ml^{-1} (Beumer and Brinkman 1989).

Of the 76 samples tested in the third series, the Gene-Trak *L. monocytogenes* assay detected 19 (25%) positives. Presence of the pathogen could be confirmed in 13 samples and, after repeated transfer in Fraser broth and subsequent streaking on the isolation media, in another three samples. This phenomenon was described earlier in a study with the Gene-Trak *Listeria* assay. The Gene-Trak protocol yielded significantly higher

confirmed positives (24) from raw milk samples than the 13 obtained with the *Listeria*-Tek ELISA or with the traditional (FDA) method (Rodriquez et al. 1993). The detection limit for the DNA-probe (6.8 $\pm$ 0.3 cfu ml^{-1}) is highest of the rapid tests investigated in this study, and slightly higher compared with those published elsewhere (Url et al. 1993, Curiale et al. 1994a). This may be contributed to the fact that the detection limits in this study were determined in presence of the competitive microflora.

In spite of its high detection level, the Gene-Trak test gives a better performance, because of its protocol. In contrast with the other methods tested, this test uses a solid medium as a second stage in the enrichment procedure. It might well be that one single *Listeria* cell on the isolation medium will multiply until a mini colony is formed, containing numbers of cells amply over the detection limit. In liquid second stage media, especially low numbers of target organisms may be inhibited by the competitive microflora, as was demonstrated for *Salmonella* by Beumer et al. (1990). In addition, it has been demonstrated that the composition of media will influence the results. For instance, the introduction of a phosphate buffer in the primary enrichment broth, improved the results of the Gene-Trak method (Bottari et al. 1995), and the optical density (OD value) obtained with cell mass from Oxford agar was higher than that with LPM (Url et al. 1993). Although evidence is lacking, *L. monocytogenes* might be present in the unconfirmed positive results. All the more, because false positive results for the Gene-Trak test in comparative studies are seldomly reported (Url et al. 1993, Bottari et al. 1995).

Because of the great variety in protocols and media used for the detection of *Listeria* it is extremely difficult to make the right choice. It has been reported in literature, that media maintaining their pH at neutral values during enrichment (UVM, Fraser broth and BLEB), perform better than those with a low buffering capacity such as LEB (Warburton et al. 1992, Beumer et al. 1996a) (Chapter 3). Theoretically, the combination of a suitable enrichment technique with a rapid test with a low detection limit, should lead to the highest number of positive results. The Tecra test (Table 1b) for the detection of *Listeria* and both the Vidas-LMO (Table 1a) and the Gene-Trak assay (Table 1c) for the detection of *L. monocytogenes* yield more than 90% of the positive samples. All three tests employ media with a high buffering capacity, and in addition the two ELISA's have low detection limits. Though the detection limit for the Gene-Trak assay is highest of all the rapid tests used in this study, its good performance may be due to the different enrichment procedure as discussed above. As expected, the ELISA with the highest

detection level (Transia), though used in combination with buffered media, had the lowest score.

To confirm positive results of rapid tests, the enrichment cultures should be streaked onto isolation media. *Listeria* colonies on the isolation media should be easy to identify, and growth of competitors should be scarce. With the performance values calculated for LPM, PALCAM and EHA, it is clear that LPM should not be used. This agrees with other studies on the efficacy of media for the isolation of *Listeria* spp. (Heisick et al. 1995, Wederquist et al. 1995).

In conclusion, in this study and in the existing literature, it was shown that the results of rapid tests depend on 1) the initial contamination of the samples with *Listeria*, 2) the composition of the media used (inhibitory agents, buffering capacity), 3) the conditions during enrichment (incubation temperature, competitors, pH), and 4) the detection limit of the test. There is enough evidence to demonstrate that high numbers of target cells in samples, in most cases lead to positive test results for rapid methods (Curiale et al. 1994a 1994b, Eckner et al. 1994). For that reason, results obtained with samples containing more than 5-10 cfu per test portion (usually 25 g), should not be emphasized in comparative studies.

Thanks to the high spiking levels (12 and 120 cfu 25 g^{-1}), the IDF method for the detection of *L. monocytogenes*, that employs a medium with a low buffering capacity, was adopted first action by AOAC International (Twedt and Hitchins 1994). Another example of using high spiking levels, resulting in a better performance of the test, is presented in reports for the AFNOR validation of the Transia test (Anonymous 1992a) and a collaborative study with this test (Anonymous 1992b). For testing the sensitivity, samples were used with contamination levels of 1−70 cfu g^{-1}. The samples used in the collaborative study contained 3, 20 and 118 cfu ml^{-1}. Moreover, the FDA method employing the poor buffered LEB, was used as reference method.

Collaborative studies (performed to be adopted first or final action by the AOAC) use both artificially and naturally contaminated samples. Because the artificially contaminated test samples are prepared for all collaborating laboratories in the same way (mixing, inoculating) in fact only information about the performance of the participating laboratories is obtained. By using reference samples (with very low numbers of the target organism), added to suspensions (1:9) of the primary enrichment broth with food samples (bought at retail outlets) more realistic information is obtained about the performance of the (rapid) tests. Following this procedure, it is inevitable that naturally

contaminated samples have to be included in the test procedure. Therefore reference samples should contain strains with typical characteristics. If available in sufficient quantities, preference should be given to naturally contaminated samples. After all, the tests are then performed under conditions in which users will apply them. However, results obtained for samples with 'low' and 'high' contamination levels should be presented for each level apart and not as an overall result. If the numbers of the target organisms in naturally contaminated samples are low, the results obtained with these samples should be emphasized.

In addition, the choice of the reference test (i.e. the enrichment and/or isolation media) should be carefully considered, because this can influence the comparison. It is recommended to use identical protocols for both rapid test and reference method. In those cases where this is impossible, cross-checking of the enrichment media is necessary. This may prevent misinterpretation of the results (Noah et al. 1991).

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6

CONFIRMATION AND IDENTIFICATION OF *LISTERIA* SPP.

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Summary

All confirmation and identification methods applied in this study can be used for the screening of suspected colonies on isolation media for *Listeria* spp. In traditional enrichment procedures the Microscreen *Listeria* latex test gives fast results. The DNA probes (Accuprobe and Gene-Trak) are very specific in detecting *Listeria monocytogenes*. For identification of *Listeria* spp. the API and Micro-ID tests performed equally well. Preference may be given to the API test, since differentiation of *L. monocytogenes* from *L. innocua* is based on the absence of arylamidase, through which tests for hemolytic activity and/or CAMP reaction can be omitted. However, the use of enhanced hemolysis agar (EHA) as isolation medium makes further testing essentially superfluous, since on this medium *L. monocytogenes* strains can be differentiated from *L. innocua*.

6

INTRODUCTION

The genus *Listeria* consists of six species: *Listeria monocytogenes*, *L. ivanovii* subspp. *ivanovii* and *londoniensis*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. grayi* (Boerlin et al. 1992, Rocourt et al. 1992). All species are ubiquitous and potential food contaminants, but only *L. monocytogenes* is a human and animal pathogen. Within the food industry there is a need for rapid methods for the detection of *L. monocytogenes*. Methodology for the isolation of listerias from food and environmental samples relies on enrichment procedures, followed by selective plating. Traditional isolation methods have been partly replaced by so-called rapid methods, of which latex tests, enzyme-linked immunosorbent assays (ELISA's) and DNA-probes are commercially available (Hofstra et al. 1994) (Chapter 5). Commercially available isolation media have no elective feature whereby *L. monocytogenes* can be distinguished from non-pathogenic listerias. Therefore typical colonies, confirmed by microscopy (Gram-positive bacilli or coccobacilli) and tests for catalase (+) and oxidase (−), have to be tested on hemolytic activity to distinguish the two species most frequently isolated, *L. monocytogenes* (hemolytic) and *L. innocua* (nonhemolytic). In further identification, traditional biochemical tests are used, including nitrate reduction and fermentation of a range of sugars (Lovett 1988). Newer methods for the confirmation of suspected colonies are based on latex tests and DNA-probes (Ninet et al. 1992). For identification on species level, systems based on biochemical tests are available (Bannerman et al. 1992, Bille et al. 1992).

In this study a latex test (Microscreen) for the confirmation of *Listeria* spp. on solid media, two DNA-probes (Accuprobe, Gene-Trak) for the confirmation of *L. monocytogenes* on solid media and two systems based on biochemical tests (Micro-ID *Listeria* in combination with a test for hemolysis, and API *Listeria*) for differentiation to species level were investigated and compared.

MATERIALS AND METHODS

Test organisms

Isolates were obtained from a study in which detection methods for *Listeria* spp. were compared (Chapter 5). From 232 samples investigated, including meat products, vegetables, cheese, egg products and environmental samples, 207 *Listeria* spp. were isolated using FDA (Lovett 1988) and USDA (McClain and Lee 1988) protocols or their modifications. The isolation media used were PALCAM (Merck 11755), lithium

chloride phenylethanol moxalactam Agar (LPM, BBL 12336) and enhanced hemolysis agar (EHA, composition and preparation as in chapter 4). Suspect colonies were purified by streaking on EHA plates and incubated for 2 d at 37°C. All *Listeria* spp. appear to be fluorescent under UV light (366 nm), in addition *L. monocytogenes* shows hemolysis. With typical *Listeria* colonies the following tests were performed: Gram reaction (Gram-positive bacilli or coccobacilli), motility (umbrella type), presence of catalase (+) and oxidase (−) (Lovett 1988) and hemolytic activity (+ or −) (Dominquez et al. 1986).

For each *Listeria* sp. a reference strain was included and *Staphylococcus aureus* and *Lactobacillus curvatus* were used as negative controls in each batch of tests.

Latex test: MicroScreen *Listeria* (Microgen, Camberley, Surrey, UK)

MicroScreen *Listeria* is a rapid latex agglutination test for both the presumptive identification of *Listeria* spp. in selective enrichment broths and confirmation of *Listeria* spp. on solid selective media. Polyvalent antisera, prepared against purified flagellin proteins from *L. monocytogenes* (antigens, A, B and C) and *L. grayi* (antigen A), are used to coat latex particles. When mixed with a suspension containing *Listeria* spp., the latex particles rapidly agglutinate to form visible clumps. The test detects all motile strains of *Listeria* spp. Purified suspect colonies were grown in brain heart infusion broth (BHIB, Difco 0037-01-6) for 20-22 h at 30°C. From each tube 50 µl was mixed with 50 µl Latex reagent on the MicroSreen slide. Agglutination within 1 min indicated the presence of *Listeria* spp. For identification from a solid medium (EHA), typical colonies were suspended in 50 µl 0.85% saline solution. If the suspension remained smooth (no autoagglutination) 50 µl of Latex reagent was placed adjacent to the saline suspension. Latex reagent and suspension were spread over the entire area and the slide was gently rocked to a maximum of 1 min. Visible clumps within 1 min indicated the presence of *Listeria* spp.

DNA-probe: Accuprobe *Listeria monocytogenes* culture identification reagent kit (Gen-Probe, San Diego, California, USA)

The Accuprobe *L. monocytogenes* culture identification reagent kit, a non-radioactive probe, is based on a hybridization reaction between a single-stranded DNA-probe and a unique sequence in the 16S rRNA region. The test was performed on overnight cultures in BHIB (20-22 h 30°C); 50 µl of these cultures was added to a tube containing the

lysis reagent. After the cells were lysed, a chemiluminescently labeled probe was added. The probe hybridizes to rRNA specific to *L. monocytogenes*. The label on the unbound probe is destroyed during the hydrolysis step, and the bound probe is detected via a chemiluminescence reaction. The light emission is measured with a luminometer (Leader 1, Berthold, Germany) and converted into relative light units (RLU). A result of >50.000 is considered as positive.

DNA-probe: Gene-Trak *Listeria monocytogenes* Assay (Gene-Trak Systems, Framingham, USA)

The Gene-Trak *L. monocytogenes* Assay is normally used for the qualitative detection of *L. monocytogenes* in food and environmental samples. In this study, the test was used for the confirmation of presumptive *L. monocytogenes* colonies on EHA. With a sterile cotton swab the entire surface of the plate was removed and suspended in 1 ml phosphate-buffered saline (Dulbecco, Oxoid BR 14a). Samples (0.5 ml) were added to test tubes, 0.1 ml pre-treatment reagent was added and the tubes were placed in a waterbath (37°C). After incubation (15 min) 0.1 ml lysis reagent was added and tubes were incubated for an additional 15 min. *L. monocytogenes* probe solution (0.1 ml) was added and in every tube a dipstick (rinsed with wash solution) was placed. Then tubes were incubated for 1 h at 65°C. Dipsticks were rinsed consecutively (1 min each) in wash solutions at 65°C and at room temperature and subsequently blotted on absorbent paper. Dipsticks were then placed in tubes with enzyme conjugate (0.75 ml) and incubated at room temperature (20 min). After rinsing in wash solution dipsticks were washed, blotted on absorbent paper, placed in tubes containing substrate-chromogen (0.75 ml) and incubated at room temperature for 30 min. Absorbance was measured at 450 nm. Assays producing absorbance values > 0.10 (read against the negative control) indicate the presence of *L. monocytogenes*.

Biochemical identification: API *Listeria* (bioMérieux, Lyon, France)

The API *Listeria* strip consists of 10 microtubes containing dehydrated substrates which enable the performance of enzymatic tests or sugar fermentations. The differentiation is based on the presence or the absence of arylamidase (DIM test, based on hydrolysis of a naphtylamide substrate), hydrolysis of aesculin, presence of α-mannosidase, and acid production from D-arabitol, D-xylose, L-rhamnose, α-methyl-D-glucoside, D-ribose, glucose-1-phosphate and D-tagatose. Suspect colonies on EHA were streaked on

tryptone soya agar (TSA Oxoid, CM 131) supplemented with 0.6% yeast extract (YE Oxoid L 21) (TSAYE). Growth was harvested in sterile 0.85% saline solution to correspond with 0.5 on the McFarland scale of standard opacities. The strip was inoculated and incubated for 20-24 h at 37°C. Addition of ZYM B reagent and reading of the strip were performed according to the manufacturer's directions.

Biochemical identification: MICRO-ID *Listeria* (Organon-Teknika, Durham, North Carolina, USA)

The MICRO-ID *Listeria* strip consists of 15 tests, including MR-VP, nitrate reduction, phenylalanine deamination, hydrogen sulfide and indole production, ornithine and lysine decarboxylation, malonate utilization, urea and aesculin hydrolysis, ß-galactosidase activity, and fermentation of xylose, rhamnose, mannitol and sorbitol. Of each strain an isolated colony from EHA was streaked on TSAYE. A suspension of each isolate was made by mixing colonies in 3.5 ml of sterile physiological saline (0.85%) to correspond to McFarland no 1 scale of standard opacities. The strip was inoculated and incubated for 20-24 h at 37°C. Addition of KOH and reading of the strip were performed according to the manufacturer's directions.

If the identification results were not identical for the two systems, fresh strips were inoculated and the traditional identification was used as described in FDA and USDA methods (Lovett 1988).

RESULTS

The results of the comparative study are shown in Table 1. All *Listeria* colonies on EHA showed fluorescence under UV light (366 nm), whereas all *L. monocytogenes* and *L. seeligeri* strains were also hemolytic.

Using the Microscreen latex test agglutination occurred with all listerias. Identical results were obtained with broth cultures in BHIB and colonies grown on EHA.

The two DNA-probes hybridized only with *L. monocytogenes*. A few strains did not hybridize with the Accuprobe or the Gene-Trak probe, although biochemical pattern and hemolytic activity indicated *L. monocytogenes*. Low numbers or other factors may be responsible for this effect, since in a second test hybridization occurred.

In some cases, reading of the strips for biochemical identification (API, Micro-ID) gave problems. In particular the test for H_2S production in the Micro-ID was very difficult to read. Any production of gray/black colour should be considered as positive.

Not in all cases did identification with API and Micro-ID give identical results. In the API test especially fermentation of ribose (5x) and in the Micro-ID test all fermentation reactions (7x) occasionally caused problems in interpretation. Reincubation of the kits for another 24 h did not improve the result. Repeated testing with the same system or with conventional fermentation tests (in culture tubes) gave clear results.

Tabel 1 Confirmation and identification of *Listeria* spp. (n=207) isolated from food and environmental samples with an immunological method (latex test), DNA probes (Accuprobe and Gene-Trak), API *Listeria* and Micro-ID *Listeria*

		Test results in					Codes read in	
***Listeria* spp.**	**N**	**EHA[a]**	**Hem.[b]**	**MS[c]**	**AP[d]**	**G-T[e]**	**API**	**Micro-ID**
L. monocytogenes								
(N=86)	79	+	+	+	+	+	6510 (3)[f]	44044 (4)
	5	+	+	+	+	+	6510	44045
	2	+	+	+	+	+	6510	44064
L. innocua								
(N=79)	61	−	−	+	−	−	7510 (5)	44044 (6)
	10	−	−	+	−	−	7110	44040
	4	−	−	+	−	−	7510	44045
	3	−	−	+	−	−	7110	44041
	1	−	−	+	−	−	3510	44044
L. grayi/murrayi								
(N=22)	14	−	−	+	−	−	7530 (1)	44047 (2)
	6	−	−	+	−	−	7530	44046
	2	−	−	+	−	−	6520	44047
L. welshimeri								
(N=18)	12	−	−	+	−	−	7711 (1)	44054 (2)
	4	−	−	+	−	−	7711	44055
	2	−	−	+	−	−	7710	44054
L. seeligeri								
(N=2)	2	+	+	+	−	−	3310	44050

[a] hemolysis on EHA, [b] hemolysis according to Dominquez et al. (1986), [c] MS= Microscreen latex test, [d] AP= Accuprobe DNA-probe, [e] G-T= Gene-Trak DNA Probe, [f] additional tested with the same system or with conventional tests

All *Listeria* reference strains and both negative controls, *St. aureus* and *L. curvatus*, showed results as expected.

Discussion

Within the total of 207 *Listeria* isolates from food and environmental samples, 86 were *L. monocytogenes* (41.5%) and 79 were *L. innocua* (38.2%). Forty-two of the isolates (20.3%) were identified as *L. grayi* (n=22), *L. welshimeri* (n=18) and *L. seeligeri* (n=2). With the exception of hemolytic activity, the phenotypic characteristics of *L. monocytogenes* are similar to those of *L. innocua*. Therefore hemolytic activity is an important property in the detection and identification of *L. monocytogenes*.

In the present study we demonstrated that with the elective features of EHA all strains were correctly identified to be *Listeria*. All isolates showing hemolytic activity were identified as *L. monocytogenes* or *L. seeligeri*. The latter occurs less frequently in food, but may be found in environmental samples. In those cases further identification may be necessary (Cox et al. 1991).

The use of isolation media other than EHA implies the use of confirmatory tests for *Listeria* spp. and or *L. monocytogenes*. It is shown in this study that the Microscreen latex test detected all *Listeria* spp. The test is easy to perform and results are available within a few minutes. Both with overnight broth cultures and colonies agglutination occurred. However, with broth cultures results were somewhat easier to read, since the cells were already suspended.

All cultures found to be *L. monocytogenes* on the basis of biochemical tests and hemolysis activity were confirmed as such with both Accuprobe and Gene-Trak DNA probes. The use of the Accuprobe is easy and rapid, whereas the Gene-Trak method, due to numerous transfer and incubation steps, is tedious and time-consuming. However, a more user friendly version is available now. Cross-reactivity with competitors (*Enterococcus* spp., *Staphylococcus* spp. and other listeria) was not observed with the Accuprobe (Bobbitt and Betts 1992, Herman and de Ridder 1993, Ninet et al. 1992) nor with the Gene-Trak DNA probe (Anonymous 1995).

With both test strips for biochemical identification of *Listeria* isolates, all strains selected for this study were identified correctly . Without additional testing the API and the Micro-ID tests agreed on 197 (95.2%) and 193 (93.2%) of the 207 cultures respectively. In other studies with these systems comparable results were obtained (Robison and Cunningham 1991, Bannerman et al. 1992, Bille et al. 1992, Higgins and

Robison 1993, Fujisawa and Mori 1994). Carbohydrate tests (mainly xylose in the API, and all fermentation test in the Micro-ID strip) were occasionally difficult to interprete. This phenomenon was also reported by Bannerman et al. (1992) and Bille et al. (1992). By repeated testing with the same system, or with conventional fermentation tests, correct results were obtained. Both test strips are easy to inoculate and read, and results are available 24 h after inoculation. Following a proposal of Rocourt et al. (1992), in the API test *L. grayi* and *L. murrayi* are considered to be a single species, *L. grayi*, whereas the Micro-ID test still differentiates between the two species.

In conclusion, all methods used in this study can be used for the screening of large numbers of strains isolated from food and environmental samples. It largely depends on the purpose of the study which method should be preferred. The use of EHA as isolation medium makes further testing essentially superfluous, since *L. monocytogenes* strains can be differentiated from *L. innocua*. However, false negatives (due to non-hemolytic *L. monocytogenes* strains) and false positives (due to presence of *L. seeligeri*) may occur. Since the isolation rate of these species is low (<1%), only in specific studies or for judicial procedures would additional tests be necessary.

In traditional enrichment procedures the Microscreen latex test gives fast and reliable results, provided that the test is applied on typical *Listeria* colonies from the isolation media used. Both DNA probes are very specific in detecting *L. monocytogenes*. The procedure of the Accuprobe is particularly convenient because of the liquid-phase hybridization, which results in fewer washing and incubation steps than in the Gene-Trak method.

In studies for identification of all *Listeria* spp. the API or Micro-ID may be used. The tests should be performed with catalase positive, oxidase negative, Gram positive bacilli or coccobacilli, showing umbrella type of motility. If not, different results may occur. Although both tests perform equally well, preference may be given to the API test, since in the API test differentiation of *L. monocytogenes* from *L. innocua* is based on the absence of arylamidase (DIM test) through which tests for hemolytic activity and/or CAMP reactions can be omitted.

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7

OCCURRENCE AND BEHAVIOUR OF *LISTERIA MONOCYTOGENES*: AN OVERVIEW

RR BEUMER

LISTERIOSIS

Human listeriosis is a rare systemic disease with a high mortality (20-30%), which occurs mostly in immunocompromized adults and newborns or infected mothers as result of a foodborne infection (Ryser and Marth 1991, Schlech 1991, Schuchat et al. 1991). The symptoms in adults are manifest primarily as meningo-encephalitis and in neonates as a severe sepsis syndrome. Listeriosis in pregnancy occurs most frequently in the third trimester. The infection of the mother may be asymptomatic or appear as a flu-like illness with fever and/or headache. The consequences for the child may be worse: spontaneous abortion, stillbirth, septicemia and meningitis (Frederiksen and Samuelsson 1992). From the survivors of listeriosis about 11% of the neonates and 30% of the adults suffer from residual symptoms (Jones et al. 1994). A milder form, with possibly a much higher attack rate, and with the symptoms of gastro-intestinal illness was described by Riedo et al. (1994). This form is probably dose dependent and does not result in invasive disease.

Most listeriosis patients (70-100%) had underlying conditions such as extreme age (very young or very old), pregnancy, cancer (often in combination with the use of immunosuppressive drugs) or AIDS (Schuchat et al. 1992, Jensen et al. 1994, Büla et al. 1995). Listeriosis is nevertheless a rare infection in AIDS patients, probably due to the antibiotic therapy and the use of a diet that excludes high risk foods. However, the disease does occur more frequently in people with AIDS than in the general population (Schuchat et al. 1991, Jensen et al. 1994). One should realize, that it is almost impossible to be certain about underlying conditions in outbreaks and sporadic cases, since these data have only partially been recorded, and often using different criteria.

The incidence of listeriosis related with pregnancy varies from 4.7-30 cases per 100.000 live births (Frederiksen and Samuelsson 1992, Jensen et al. 1994, Jones et al. 1994). The annual incidence of sporadic cases is 7.4 per million (Schuchat et al. 1992). The incidence of listeriosis among HIV-infected persons and AIDS patients is 52 and 115 cases per 100.000 respectively (Jurado et al. 1993).

In 1960 it was already known that in view of the proved pathogenicity of *L. monocytogenes*, there was a risk of listeriosis for all professional groups dealing with animals or animal products, meat, game, non-pasteurized milk and milk products in particular. Based on increase in cases of human listeriosis from 20 in the period up to 1945 to almost 1000 in the period 1945-1960, it was recommended to pay more attention to food hygiene and to food legislation to prevent listeriosis (Seeliger 1961).

The first described large outbreak of human listeriosis occurred in Halle (Germany, 1949-1957) after consumption of raw milk and milk products (Seeliger 1961). Despite the appeal to a better food hygiene, other large outbreaks have been reported thereafter. The outbreaks in the United States and Canada in 1979-1985 were strongly associated with the consumption of cold stored foods such as coleslaw (Schlech et al. 1983), pasteurized milk (Fleming et al. 1985) and Mexican-type cheese (Linnan et al. 1988). These outbreaks resulted in numerous reports dealing with the isolation of this pathogen from food, feed and environmental samples and its behaviour in food products and various environments (Farber and Peterkin 1991, Ryser and Marth 1991). In Europe, outbreaks were observed from 1983 onward and these were mainly related to soft cheese (Bille 1989), pâté (McLauchlin et al. 1991), pork tongue in aspic (Goulet et al. 1993), potted pork (Anonymous 1993) and raw milk soft cheese (Goulet et al. 1995).

For sporadic cases it is more difficult to determine the source of the causative organism. Foodborne transmission could be demonstrated in some cases (Schuchat et al. 1992), as well as occupational disease of farmers and veterinarians (McLauchlin and Low 1994), and hospital infections (McLauchlin 1996). In case control studies of dietary risk factors it was shown that soft cheeses and/or food obtained from delicatessen counters (Schuchat et al. 1992), consumption of raw milk and/or pâté (Jensen et al. 1994) were associated with listeriosis.

Almost all cases of human listeriosis are due to *L. monocytogenes* (McLauchlin 1990a, 1990b), although *L. seeligeri* and *L. ivanovii* (Cummins et al. 1994, Lessing et al. 1994) have also been implicated incidentically. Caution is needed by assuming that the infective dose for both non-foodborne and foodborne listeriosis is high. Because of the relatively small number of outbreaks and sporadic cases detailed information is not available.

CONTAMINATION OF FOOD PRODUCTS WITH *LISTERIA*

The presence of *Listeria* spp. in sewage and faeces, and the fact that these micro-organisms readily multiply in decaying vegetable material, explain its occurrence in soil. Just as in human faecal carriage there was a seasonal variation in *Listeria* isolation from garden soils: in most samples listerias were detected in summer months. *L. seeligeri* and *L. ivanovii* were the most frequently isolated species. Factors such as organic fertiliser, the presence of pets and plants or the isolation method used may be responsible for this observation (MacGowan et al. 1994). Thus, it is likely that most raw food products will

harbour listerias in low numbers. Fish and shellfish may be contaminated by water, vegetables by soil or water, milk by poor hygiene during milking, and meat, game and poultry by contact of the carcasses with faeces during slaughter. In vegetables, on surfaces where the cell tissue has lost its integrity and decay becomes visible, high numbers may be expected. In Table 1 a survey is given of the primary contamination of raw food products with *Listeria*.

Table 1 Incidence of *Listeria monocytogenes* in raw food products

Product	# Samples	*Lm*[a](%)	Others[b] (%)	References
Raw meat	175	31 (18)	111 (63)	Sheridan et al. 1994
Minced meat	168	29 (17)	113 (67)	DeSimón et al. 1992
Poultry	100	60 (60)	28 (28)	Pini and Gilbert 1988
Fish	382	48 (13)	9 (2)	Masuda et al. 1992, cited in: Ben Embarak 1994
Mussels	175	0 (0)	2 (1)	Decastelli et al. 1993
Bivalve molluscs	40	3 (8)	6 (15)	DeSimón et al. 1992
Cows' milk	9837	342 (3)	221 (2)	Ryser and Marth 1991
Cows' milk	160	25 (16)	NR	Fenlon et al. 1995
Raw eggs	173	78 (45)	47 (27)	Moore and Madden 1993
Vegetables	103	8 (8)	11 (11)	DeSimón et al. 1992
Vegetables	194	3 (3)	NR[c]	Heisick et al. 1989
Potatoes	132	28 (21)	NR	Heisick et al. 1989
Radish	132	19 (14)	NR	Heisick et al. 1989

[a] *Lm* = *Listeria monocytogenes*, [b] Others = other *Listeria* spp., [c] NR = not reported

L. monocytogenes can be found particularly on products of animal origin. Its presence on processed, ready-to-eat foods, is usually the result of contamination from the production environment (Cox et al. 1989). Prevalence has been demonstrated in numerous products, including meat products (Farber and Daley 1994), fish products (Jemmi

1993), dairy products (Loncarevic et al. 1995) and ready-to-eat food products (Wilson 1995).

Growth of *L. monocytogenes* on these products is dependent on many factors. These include intrinsic factors (e.g. pH, water activity (a_w), and presence of preservatives), extrinsic factors (storage temperature and gas atmosphere composition) and implicit factors (synergism or antagonism with competitive microorganisms). Microbiologically stable food products can be obtained by removal of the microorganisms (filtration), growth inhibition of the microorganisms (refrigeration, freezing, decreasing of pH and/or a_w, adding preservatives or combinations of these factors), and inactivation of microorganisms (pasteurization, sterilization, irradiation).

Though *L. monocytogenes* is a robust microorganism in terms of its ability to grow under the conditions commonly found in foods, its ability to multiply at refrigerator temperatures is probably the most important factor for the presence of high numbers in non-sterile food products with a long shelf life. In all substrates ($a_w > 0.92$ and $4.5 <$ pH < 9) growth was observed from 0-45°C with generation times varying from 5-7 days at 0°C on beef (Grau and VanderLinde 1990) to 52 min in tryptone soya broth at 45°C (Petran and Zottola 1989).

To meet the consumers' demand for wholesome and 'natural' convenience foods, cook-freeze, cook-chill and minimally processed chilled foods have been developed. The potential for these foods to serve as vehicles for foodborne disease depends on the source and handling of the product as well as on the consumer (Hedberg et al. 1994).

For some preservation methods characteristics are shown in Table 2. It is clear that only heat treatment and the less common irradiation treatment will result in *Listeria*-free products. As contamination levels with *Listeria* are usually low ($< 10^2$ g^{-1}), pasteurization (heating to a core temperature of 71°C) is sufficient to inactivate *Listeria* (Mackey et al. 1990). Chemical and biological preservation only have a limited activity spectrum (Holzapfel et al. 1995). Moreover, resistance of *Listeria* (and other microorganisms) to these agents has often been reported (El-Gazzar and Marth 1991, Davies and Adams 1994).

L. monocytogenes is extremely resistant to high concentrations of NaCl both in broth (McClure et al. 1989) and in food products (Hudson 1992, Peterson et al. 1993). In combination with other factors (low pH and refrigeration temperature) no growth was observed (Cole et al. 1990). In a study of Houtsma et al. (1993) it was shown that *Listeria* spp. were inhibited more readily by sodium lactate than by sodium chloride.

Fermented foods traditionally have been considered to be pathogen free due to various combinations of inhibitory factors such as low pH, high levels of salt and a low storage temperature. *L. monocytogenes* has been shown to survive the manufacture and storage of fermented foods made from raw materials contaminated with this microorganism. These products include a wide variety of cheeses and fermented milks (Pearson and Marth 1990) and fermented meats (Johnson et al. 1988). Apart from the use of effective fermentation, a potential means of controlling pathogens is through antimicrobial com-

Table 2 Growth and survival characteristics for *Listeria* spp.

Food product	Factor	Effect	Reference
Minced meat	-18°C	Survival for 6 months	Kaya and Schmidt 1989
Ground meat	60°C	D-value 3.1 min	Farber 1989
Chicken breast	60°C	D-value 8.7 min	Mackey et al. 1990
Chicken breast	65°C	D-value 0.5 min	Mackey et al. 1990
Raw milk	58°C	D-value 5.0 min	Bradshaw et al. 1987
Raw milk	63°C	D-value 28 s	Bradshaw et al. 1987
Raw milk	66°C	D-value 7 s	Bradshaw et al. 1987
Raw milk	72°C	D-value 1 s	Bradshaw et al. 1997
Minced meat	ionizing radiation	D-value 1.1 kGy, -18°C	Stegeman 1988
Minced meat	ionizing radiation	D-value 0.4 kGy, 4°C	Stegeman 1988
Chicken meat	ionizing radiation	D-value 0.5 kGy, 4°C	Kamat and Nair 1995
Fish	sorbate 30 mg kg^{-1}	Extended lag phase	Dorsa et al. 1993
Smoked fish	NaCl 6%	Some growth inhibition	Peterson et al. 1993
Cabbage lysozyme	100 mg kg^{-1}	Declined 100-fold	Hughey et al. 1989
Fish lysozyme	3 mg ml^{-1} water	Extended lag phase	Wang and Shelef 1992
Minced meat	thyme oil 0.1%	100-fold reduction	Aureli et al. 1992
Turkey skin	nisin 100 IU ml^{-1}	10-fold reduction	Mahadeo and Tatini 1994
Cream	bacteriocin PA-1	MIC[a] 55 AU[b] ml^{-1}	Pucci et al. 1988

[a] MIC = minimum inhibitory concentration, [b] AU = arbitrary unit: 5 ml of the highest dilution of filtrate yielding a definite zone of growth inhibition on the indicator lawn

pounds produced by lactic acid bacteria. Application of these so-called protective cultures should be considered only as an additional measure to good manufacturing processes, because its effectiveness, tested in different types of food products, is limited to a reduction in initial numbers of *L. monocytogenes* (Holzapfel et al. 1995). Though there are reports on the involvement of lactic acid bacteria (LAB) in human infections (Aguirre and Collins 1992), an European Union-sponsored workgroup concluded that, with the exception of enterococci, the overall risk of LAB infection is very low (Adams and Marteau 1995).

Nisin was affirmed GRAS (generally recognised as safe) by the Food and Drug Administration (FDA) and has an approval for its use in pasteurized cheese spreads to inhibit the growth of clostridia (Food and Drug Administration, 1988) and is now used as a biopreservative in 57 countries around the world (Hurst and Hoover 1993).

Nisin-producing strains of *Lactococcus lactis* subsp. *lactis* were first employed by Hirsch et al. (1951) to inhibit gas-blowing by anaerobic sporeformers in Swiss-type cheese. Two decades later, Lipinska (1971) used nisin-producing strains in combination with nisin-resistant ones, possessing desirable cheese making properties, for the same purpose. Recently, some authors (Harris et al. 1989, Sulzer and Busse 1991) have revived interest in nisin by showing that this bacteriocin exerts a bactericidal effect towards *L. monocytogenes* both in culture media and in some types of cheeses. In Camembert cheeses made with a nisin-producing starter, highest nisin concentrations (up to 700 IU g^{-1}) were found in curd (9 h after adding the starter). Then concentrations decreased slowly during the next 9-24 h and very fast during ripening. In the presence of nisin, the numbers of *Listeria* decreased until the end of the second week of ripening, leading to a reduction with ca 3 log units. Thereafter, regrowth occurred in Camembert cheeses, sooner on the surface than in the core. Nisin was particularly effective when cheese milk contained low numbers (10^1-10^3 cfu ml^{-1}) of this microorganism (Maisnier-Patin et al. 1992). The application of nisin in meat products (turkey) resulted also in a decrease of the initial population of *L. monocytogenes* (Mahadeo and Tatini 1994). For other bacteriocins, produced by LAB in fermented sausages (Foegeding et al. 1992), frankfurters (Berry et al. 1991) and ready-to-use salads (Lichter 1993 In: Holzapfel et al. 1995) similar results were reported. As the activity spectrum of bacteriocins is restricted to closely related microorganisms, Gram-negative pathogens and spoilage bacteria will not be inhibited. Moreover, it should be recognized that bacteriocin-

resistant strains of *L. monocytogenes* may arise due to prolonged or repeated exposure to bacteriocin in food production systems (Harris et al. 1991, Davies and Adams 1994).

Since contradictory results on the growth of *L. monocytogenes* on raw meat surfaces have been published, the factors that influence the growth of this pathogen on these products are investigated in Chapter 8. In processed meats with a long shelf life, listerias can grow to unacceptably high levels. In Chapter 9 this is shown for the growth of *L. monocytogenes* on ham, luncheon meat and chicken breast. The effect of the competitive microflora, lactic acid bacteria, is also described in this chapter.

Osmoprotectants such as betaine, a trimethyl amino acid, and proline are involved in the adaptation to high extracellular solute concentrations. Another trimethyl amino acid is carnitine, an important compound in mammalian tissues, involved with lipid metabolism. Chapter 10 describes the effect of proline, betaine and carnitine on the growth of *L. monocytogenes* and *L. innocua* in a minimal medium and in a rich medium with increasing salt concentrations.

Especially in sporadic cases of listeriosis it is difficult to link food products with this disease. It may be possible that other sources of *L. monocytogenes* might be responsible for human listeriosis. The occurrence of this pathogen in domestic environments is reported in Chapter 11. The general discussion, including recommendations for standards and detection of this pathogen, is presented in Chapter 12.

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8

GROWTH OF *LISTERIA MONOCYTOGENES* ON RAW MEAT

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This chapter has been submitted

SUMMARY

Growth of *Listeria* on raw meat may be affected by storage temperature, pH, water activity, availability of (micro)nutrients, presence of inhibitory substances and competitive microorganisms. In this study sterile raw beef, veal, pork and chicken meat and buffer suspensions of those meats were inoculated with eight strains of *Listeria monocytogenes*. Growth of the test strains on raw meat was strain dependant and mainly influenced by initial pH and storage temperature. At 7°C, addition of compounds, essential for the growth of these organisms in a minimal medium (glucose, amino acids, vitamins, and various iron sources), to the raw meat, never resulted in growth of the test strains on beef, veal and pork within two weeks.

8

INTRODUCTION

Listeria monocytogenes is isolated from beef, pork, lamb and poultry carcasses and from the corresponding minced meats. The presence of this pathogen on carcasses has been attributed to contamination by faeces, hides, pelts and the slaughter environment. The incidence of *Listeria* on meat varies from 0 to 94%, influenced by many factors such as type of meat, rearing and slaughtering hygiene. The coexistence of several *Listeria* spp. on the same meat sample has often been reported. The prevalence of *L. monocytogenes* in meat samples contaminated with *Listeria* varied from 0-68%, with pork and poultry more commonly contaminated than beef or lamb. In raw processed meat, such as minced meat, the prevalence of *Listeria* was higher, however, only in exceptional cases numbers exceeded 10^2 cfu g^{-1} (Johnson et al. 1990, Farber and Peterkin 1991).

Several studies have reported conflicting results concerning the survival and growth of *L. monocytogenes* on meat. Due to the use of different strains (Barbosa et al. 1995), inoculated on various types of meat, and stored at temperatures varying from 0-30°C under different atmospheres, results cannot be compared directly (Johnson et al. 1988, Buchanan and Klawitter 1991, Kaya and Schmidt 1991). It was shown that both pH and incubation temperature influenced the lag time and growth rate on beef lean tissue (Kaya and Schmidt 1991, Grau and Vanderlinde 1993). It has also been suggested that fatty acids (Gouet et al. 1978, Wang and Johnson 1992) or other substances (Buchanan and Klawitter 1991) have an inhibitory effect towards *L. monocytogenes*. However, in another study quite the contrary was found for fatty acids (Grau and Vanderlinde 1993).

Effects of competitive microorganisms have also been described: growth restriction by reduced pH or production of bacteriocins or other inhibitors due to the rapid growth of lactic acid bacteria. Contrarily, growth stimulation of *Listeria* by proteolytic activities of *Pseudomonas* spp. on meat, has also been reported (Gouet et al. 1978, Farber and Peterkin 1991, Kaya and Schmidt 1991).

Other studies have suggested that the growth of *L. monocytogenes* on meat is affected by the availability of (micro)nutrients (Farber and Peterkin 1991). It is likely that all (micro)nutrients for the growth of *L. monocytogenes* are present in raw meats, because its growth on processed meat products is well-documented (Beumer et al. 1996) (Chapter 9). Maybe an exception should be made for the availability of iron: after heat treatment there is an increase in non-haem iron and a concomitant decrease in haem iron (Schricker et al. 1982). If *Listeria* prefers the uptake of non-haem iron, the availability of iron could be a decisive factor for the growth of *L. monocytogenes* on raw meat.

The objectives of this study were to determine whether lack of nutrients (iron, glucose and/or amino acids), temperature, or pH (or combinations of these factors) influence the growth of *L. monocytogenes* on sterile beef.

MATERIALS AND METHODS

Minimal medium

A chemically defined minimal medium (MM) based on that described previously by Premaratne et al. (1991) was employed. Its composition (amount per liter): KH_2PO_4 6.56 g, Na_2HPO_4 30.96 g, $MgSO_4$ 0.41 g, ferric citrate 0.088 g, glucose 10.0 g, L-leucine 0.1 g, L-isoleucine 0.1 g, L-valine 0.1 g, L-methionine 0.1 g, L-cysteine 0.1 g, L-glutamine 0.6 g, riboflavine 0.5 mg, thiamine 1.0 mg, biotin 0.5 mg, and thioctic acid 0.005 mg.

To investigate uptake of iron, Fe(II)sulphate (Merck 3965, 237 mg 50 ml^{-1}), $Fe(III)Cl_3$ (Merck 803945, 138 mg 50 ml^{-1}), Fe(III)citrate (Merck 3862, 250 mg 50 ml^{-1}), Fe(III)nitrate (Merck 3883, 344 mg 50 ml^{-1}) were each added to 9 ml MM (without Fe-citrate) as filter sterilized solutions to a final concentration of 1.7 mM Fe. For hemin (Sigma H-2250) and hemoglobin (Sigma H-2500) final Fe concentrations in MM were 0.61 mM and 39 μM respectively.

Raw meat

Raw meat (veal, beef, pork, chicken) was bought directly after slaughtering from a local butcher and sterilized by dipping in 3% peracetic acid (Labadie et al. 1977). Thereafter the meat was rinsed with sterile water and on all sides 3-4 cm was cut off using a sterile knife for each slice. This procedure was repeated and thereafter the meat was divided into pieces of about 10 cm^2 (thickness 1 cm). Each slice of meat was transferred to a slide, resting upon two glass rods (Ø 10 mm) in a sterile Petri dish with 8 ml sterile water to prevent drying out.

Suspensions of sterile raw meat with buffer solutions

Twenty grams of sterile meat (veal, beef, pork, chicken) were mixed in a stomacher with McIlvain buffers (20 ml) to obtain initial pH-values of 5.2, 5.4, 5.6, 5.8, 6.0 and 6.2. Double strength brain heart infusion broth (BHIB, Difco 0037-01-6) mixed with equal volumes of the McIlvain buffers were used as controls (10 ml/tube, sterilized 15 min/121°C).

Inoculum preparation

All *Listeria* strains were isolated from food or food production environments. They included *L. monocytogenes* serotype 1/2a (strain An10A, 106 and 294), *L. monocytogenes* serotype 1/2b (strain 1), *L. monocytogenes* serotype 3a (strain 311) and *L. monocytogenes* serotype 4b (Scott A, strain 144 and L4492). Each strain was grown in 10 ml BHIB at 30°C for 20-22 h. The cells were harvested by centrifugation (2600 g for 15 min) and resuspended in MM. This procedure was repeated and the cells were resuspended in MM to give approximately 10^9 cells ml^{-1}. This suspension was used to inoculate MM (0.1 ml in 10 ml), meat slices, meat suspensions and BHIB (0.1 ml of the 10^{-6} dilution), resulting in an inoculum level of 10^6 per ml MM, 10^1 per g meat, 2.5 per ml meat suspension and 10^1 per ml BHIB. Tubes with MM were incubated 30 h at 37°C and growth was measured spectrophotometrically by determining the optical density at 660 nm (OD_{660}) (Beckman Instruments, Mijdrecht, Holland). Meat slices were incubated for two weeks at 7 and 15°C. Suspensions of raw meat and buffered BHIB were incubated for two weeks at 7°C. Samples were taken after various time intervals and the levels of *Listeria* and total counts were determined as described below. The pH of buffered BHIB and meat suspensions was measured in the primary dilution.

Microbiological sampling

Meat samples were homogenized with a stomacher (2 min). Appropriate dilutions were plated out on enhanced hemolysis agar (EHA, composition and preparation as in chapter 4) and tryptone soya agar (TSA, Oxoid CM 131) and incubated for 2 days at 37°C. Colonies on EHA showing fluorescence under UV light (366 nm) and a zone of hemolysis were considered to be *L. monocytogenes*.

RESULTS AND DISCUSSION

Uptake of iron

Highest OD values were obtained with Fe(III)-citrate and in descending order with Fe(III)-chloride, Fe(II)-sulphate, Fe(III)-nitrate, hemoglobin and hemin. The stimulating effect of Fe(III)-citrate on the growth of *L. monocytogenes* was reported in previous studies (Cowart and Foster 1985, Adams et al. 1990, Premaratne et al. 1991). Contradictory results have been published about the uptake of Fe(III)-chloride. According to Adams et al. (1990) and Premaratne et al. (1991) *L. monocytogenes* was not able to use this component as iron source. Our results agree with those of Trivett and Meyer (1971)

Tabel 1 Growth of *Listeria monocytogenes* on sterile beef, veal, pork and chicken meat at 7°C

L. monocytogenes serotype, strain	Beef D15−D01[a]	Veal D15−D01	Pork D15−D01	Chicken D15−D01
1/2a, 106	+ 0.1	− 0.1	+ 0.2	+ 2.6
1/2a, 294	+ 0.1	− 0.1	+ 0	+ 3.1
1/2b, 1	− 0.1	+ 0.1	+ 0.2	+ 3.2
3a, 311	− 0.2	+ 0.1	+ 0.2	+ 3.2
4b, 144	− 0.1	− 0.2	+ 0.1	+ 3.4
4b, L44-92	− 0.1	− 0.2	− 0.2	+ 3.3
pH in primary dilution	+ 0.1	+ 0	+ 0.2	+ 0.2

[a] Difference between log counts of *L. monocytogenes* or pH value on the first (D01) and the last day (D15) of the storage time

and Vidon and Spreng (1992), who reported the uptake of Fe(III)-chloride by *L. monocytogenes*. The stimulating effect of Fe(II)-sulphate and Fe(III)-nitrate on the growth of *L. monocytogenes* agrees with previous studies on the effect of Fe(II)-ammonium sulphate (Adams et al. 1990) and Fe(III)-nitrate (Premaratne et al. 1991). Addition of hemoglobin and hemin resulted in increased growth of *L. monocytogenes* in the MM, however, effects were less obvious than observed with the iron salts. Our results were comparable with the findings reported for hemoglobin (Deneer and Boychuk 1993) and hemin (Premaratne et al. 1991).

Growth on sterile beef, veal, pork and chicken

Sterile slices of meat were inoculated with test strains of *L. monocytogenes* (10^1 cfu g^{-1}) and stored for 15 days at 7°C. Microbial counts were performed after 1, 3, 8 and 15 days (Table 1). On beef, veal and pork no growth of the test strains was observed at 7°C. For all strains an increase in numbers (2.6-3.4 log units) was observed on chicken meat. The pH of the sterile meat slices slightly increased with storage time (0.1-0.2 unit), which agrees with the findings of Manu-Tawiah et al. (1993).

In an additional experiment, where sterile beef, veal and pork were inoculated with increased serial inocula (10^3 to 10^6 cfu g^{-1}) of the test strains, again no growth was observed. This might be due to a lack of available nutrients in beef, veal and pork. However, simultaneous addition of the essential compounds of the MM (glucose, amino acids, vitamins, various iron sources) to the various types of meat, apart or in various combinations at concentrations used in the MM, never resulted in growth of the test strains during the storage time of 15 days (data not shown).

It seems more likely that growth of *L. monocytogenes* on the meat was inhibited by the rather low pH values of beef, veal and pork, in combination with the low storage temperature (7°C). Therefore, sterile beef and pork were inoculated with *L. monocytogenes* (strain 1 and 106) and stored at 15°C. Both in beef and pork growth of the test strains was observed. The difference between the log counts on the first and last day (day 15) of the storage time of beef were +3 and +3.6 for strain 106 and strain 1, respectively. A slightly better growth, +3.6 and +3.8, was observed on pork meat for strains 106 and 1, respectively. This was probably due to the difference in pH of pork meat (pH 5.6-5.8) and beef (pH 5.3-5.5).

Growth in BHIB and meat suspensions

To determine whether the low pH values of the meat were responsible for the growth inhibiting effect of the test strains, sterile meat samples were mixed with sterile buffers, inoculated with *L. monocytogenes* and incubated at 7°C. The results for three strains are presented in Table 2. During the first week of storage the initial pH values of the meat suspensions shifted towards the pH values of the meat. For chicken and pork this resulted in three and for beef in two pH-clusters. During the second week of storage pH values did not change significantly. Therefore, growth of the test strains is reported as the difference in numbers after storage for one and two weeks. Strikingly, considerable variations between strains were observed. *L. monocytogenes* An10A did grow at all pH values in BHIB (5.2-6.2), whereas for *L. monocytogenes* L4492 and *L. monocytogenes* Scott A growth was shown from pH 5.4 and pH 5.6, respectively. For all test strains the growth on meat was comparable with the growth in BHIB. The growth yields in BHIB were slightly, however, not significantly higher compared with these in meat suspensions with corresponding pH values. This may be due to the limiting concentration of nutrients, as shown for glucose by Drosinos and Board (1994). However, because the numbers after two weeks at 7°C both in meat suspensions and in BHIB did

Table 2 Growth of *Listeria monocytogenes* (strain An10A, L4492 and Scott A) in suspensions of meat with buffer solutions and in buffered brain heart infusion broth (BHIB) at 7°C

Type of meat	Initial pH	pH, 1 week	pH, 2 weeks	Growth (logW2-logW1)[a]		
				An10A	L4492	Scott A
Chicken	5.2	5.6 ± 0.04	5.6 ± 0.02	2.7	2.6	1.8
	5.4					
	5.6	5.7 ± 0.05	5.7 ± 0.04	2.8	2.6	1.9
	5.8					
	6.0	5.9 ± 0.04	5.9 ± 0.03	2.9	2.5	2.7
	6.2					
Pork	5.2	5.6 ± 0.04	5.6 ± 0.04	2.7	2.5	1.7
	5.4					
	5.6	5.8 ± 0.05	5.8 ± 0.05	2.7	2.8	2.0
	5.8					
	6.0	5.9 ± 0.04	5.9 ± 0.02	2.8	2.9	2.8
	6.2					
Beef	5.2	5.4 ± 0.05	5.4 ± 0.05	0.6	0.4	0.0
	5.4					
	5.6					
	5.8	5.5 ± 0.06	5.5 ± 0.04	0.6	0.7	0.0
	6.0					
	6.2					
BHIB	5.2	5.2 ± 0.03	5.2 ± 0.02	0.3	0.0	0.0
	5.4	5.4 ± 0.02	5.4 ± 0.05	0.8	0.6	0.0
	5.6	5.6 ± 0.04	5.6 ± 0.04	2.9	3.1	2.0
	5.8	5.8 ± 0.02	5.8 ± 0.04	3.0	3.1	2.4
	6.0	6.0 ± 0.03	6.0 ± 0.02	3.1	3.0	2.4
	6.2	6.2 ± 0.02	6.2 ± 0.02	3.1	3.0	2.3

[a] Difference between log counts of *L. monocytogenes* at the end of the first week and at the end of the second week of storage time

not exceed 10^4 cells ml^{-1} and a competitive microflora was absent, other factor(s) may be responsible for that.

In summary, the growth of *L. monocytogenes* on meat is strain dependant and influenced by temperature, pH, water activity (a_w), availability of (micro)nutrients and

presence of inhibitory substances and competitive microorganisms. After temperature, a_w and pH are the most important factors for growth. Fresh meats have an a_w which is usually about 0.99 and because growth of *L. monocytogenes* has been reported in meat model systems down to levels as low as 0.96 (Chen and Shelef 1992), a_w will not be a restricting factor for the growth of *Listeria* on meat, unless the surface is subject to desiccation. The pH values at which growth of *Listeria* occurs depend, amongst others, on temperature. Increasing temperatures will result in growth at lower pH values (up to pH 4.4) in culture media (George et al. 1988). Though contradictory results are published about the effect of temperature on growth of *Listeria* on low pH meat (Avery et al. 1994), from our data it is clear that storage of low pH meat at abuse temperatures (15°C) will result in growth of *L. monocytogenes*, whereas no growth was observed at 7°C. Lack of nutrients is not an obvious reason for that, because all amino acids and glucose are present in meat. Glucose is present in rather low (max. 0.01%) concentrations (Belitz and Grosh 1987) and partly as glucose-6-phosphate, which cannot be catabolized by *L. monocytogenes* (Drosinos and Board 1994). However, addition of glucose, iron and amino acids to beef did not result in growth during storage time at 7°C. Longer incubation times (several weeks) could result in growth of the test strains at 7°C on beef and pork, since it has been shown that the pH-range for growth of *Lactobacillus curvatus* in broth is independant of the incubation temperature (Wijtzes et al. 1995).

In natural ecosystems various microbial populations compete for the nutrients available. This was demonstrated for the growth of *L. monocytogenes* in meat juice. Growth of this pathogen ceased with glucose depletion (Drosinos and Board 1994) and its subsequent die-off was probably associated with the production of acetic acid by the competitive microflora. Probably, the growth of *L. monocytogenes* on meat surfaces is determined rather by diffusion than depletion of the nutrients, or antibiosis by competitors. Though lactic acid bacteria are antagonistic toward *L. monocytogenes* (Raccach et al. 1989), it is questionable if they can prevent surface growth of *Listeria*. During the storage of cooked meat products, lactic acid bacteria (including bacteriocin producing strains) only slightly inhibited growth of *L. monocytogenes*. At the end of the shelf life (4-6 weeks at 7°C) levels reached were still 10^7 cfu g^{-1} (Beumer et al. 1996).

In conclusion, our results show that growth of *L. monocytogenes* on low pH meat is mainly influenced by initial pH and storage temperature. Temperature abuse during storage may result in the growth of this pathogen. In such situations it is difficult to

predict to what extent competitive microorganisms, or packaging conditions (e.g. modified atmosphere packaging), are inhibitory to the growth of *L. monocytogenes*. For that reason, and because survival at low pH for a long period is likely (Farber and Peterkin 1991), this raw meat should preferably not be used for the production of ready-to-eat products such as steak tartare.

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9

GROWTH OF *LISTERIA MONOCYTOGENES* ON SLICED COOKED MEAT PRODUCTS

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SUMMARY

During the shelf life (4-6 weeks) of artificially contaminated sliced cooked meat products such as luncheon meat, ham and chicken breast, the growth of *Listeria monocytogenes* in vacuum packaged products was similar to the growth in modified atmosphere (30% CO_2/70% N_2) packaged products. The presence of competitors (lactic acid bacteria), even in concentrations one hundred times those of *L. monocytogenes*, inhibited growth of this pathogen only slightly. At the end of the shelf life, levels were still 10^7 cfu g^{-1}. Due to the lower initial contamination, levels in naturally contaminated products were about 10^4 cfu g^{-1}. To prevent outgrowth of *L. monocytogenes* to such high levels it is necessary to prevent recontamination during slicing and packaging, and to shorten the rather long shelf life of these products. Due to the low pH of fermented sausage (saveloy) and raw ham (Coburger), the numbers of *L. monocytogenes* decreased below the detection level.

9

INTRODUCTION

Listeria monocytogenes has been isolated from a wide variety of foodstuffs, including raw and cooked meat products. Conflicting results have been published about the growth of this pathogen on raw meat products. Nevertheless, growth appears to be highly dependent on pH, temperature, type of tissue and the competitive microflora (Farber and Peterkin 1991) (Chapter 8). In cooked meat products *L. monocytogenes* should be absent. The heating of these products for 2 min at 70°C, or to core temperatures of 71°C, is likely to inactivate any *L. monocytogenes* present (Gaze et al. 1989, Mackey et al. 1990).

Although reports on survival of *L. monocytogenes* during processing have been published regularly, evidence is often obtained by heating meat products with unrealistic high levels (10^7-10^8 cfu g^{-1}) of added listerias (Boyle et al. 1990, Michel et al. 1991, Hardin et al. 1993). The presence of heat-stressed cells is probably only of interest when cells have been exposed to sublethal temperatures for short periods of time, since this significantly increases the heat resistance (Linton et al. 1992). However, these results were also obtained in experiments using high initial levels of *Listeria*.

Because raw meats generally contain low numbers of *Listeria* (less than 100 cfu g^{-1}) (Sheridan et al. 1994), the decimal reductions obtained with the heating processes normally applied are sufficient to ensure safe products (Mackey et al. 1990). This may be supported by the findings of Wang and Muriana (1994). They found *Listeria* spp. only in the liquid exudate of retail frankfurters and not in the internal meat, indicating that the presence of *Listeria* was most likely due to postprocess contamination. The incidence of *L. monocytogenes* on prepacked sliced cooked meat products has been reported by a number of workers (McLauchlin and Gilbert 1990, Grau and Vanderlinde 1992, Pinner et al. 1992, Sheridan et al. 1994). As these products are supposed to have a refrigerated shelf life of several weeks, it is important to know the growth potential of this pathogen on cooked meat products. From previous work it is known that luncheon meat, ham and cooked chicken breast were the most frequently contaminated cooked meat products in the Netherlands (De Boer 1990). In this study, these products, and two raw products (saveloy and Coburger ham), were artificially contaminated with low numbers of *L. monocytogenes*. The samples were packaged under modified atmosphere, stored at 7°C and within the shelf life of these products (4-6 weeks), the extent of growth of *L. monocytogenes* and competitors (lactobacilli) was determined.

MATERIAL AND METHODS

Bacterial strains and inoculum preparation

L. monocytogenes, which had been isolated from sliced roast beef, was grown in brain heart infusion broth (BHIB, Difco 0037-01-6). *Lactobacillus curvatus* (isolated from luncheon meat) and *Lactococcus lactis* (a nisin producing strain from our collection), were grown in de Man, Rogosa and Sharpe broth (MRSB, Oxoid CM 359) at 30°C. After 20-24 h serial dilutions were made in peptone physiological saline solution [PPSS, composition: NaCl (8.5 g l^{-1}) and neutralized bacteriological peptone (Oxoid L 34, 1 g l^{-1})]. To check the inoculum level appropriate serial dilutions were plated onto tryptone soya agar (TSA, Oxoid CM 131) for *Listeria* and onto MRS-agar (Oxoid CM 361) for lactic acid bacteria. Plates were incubated 2-3 d at 30°C.

Meat products and inoculation

Sliced cooked meat products (luncheon meat, ham and chicken breast), fermented sausage (saveloy) and raw ham (Coburger) were transported 1-3 days after production in insulated cool boxes to the laboratory. The packages were opened aseptically and the contents were transferred, after removing the upper slice, into sterile vacuum bags (polyamide/polyethylene). The meat products were inoculated (on the surface or between two central slices) with 100 μl of the 10^4 inoculum, resulting in an inoculum level of 10 cfu g^{-1}. In some experiments the surface of the meat products was also inoculated with 100 μl of 10^3-10^5 inocula of *Lb. curvatus* or *Lc. lactis*, to investigate inhibitory effects on the growth of *L. monocytogenes*. With a sterile Drigalsky the suspension was spread over the surface. Directly after inoculation, the meat products were packaged in 30% CO_2/70% N_2 or vacuum, using an tabletop apparatus for vacuum and modified air packaging, type ALVAC I-90 (Stephan, Almelo, Netherlands). The inoculated products and control samples (uninoculated products in original packages and in vacuum bags) were stored at 7°C until the end of the shelf life indicated by the manufacturer.

Microbiological investigation

After 1, 4, 7, 11, 14, 28 and 37 days the levels of *L. monocytogenes* were determined on enhanced hemolysis agar (EHA, composition and preparation as in chapter 4). All *Listeria* spp. appear to be fluorescent under UV light (366 nm), in addition, *L. monocytogenes* shows hemolysis. Presumptive *Listeria* colonies from control samples were

purified by streaking on a non-selective medium (TSA) and incubated for two days at 30°C. With colonies demonstrating a characteristic blue colour under Henry illumination, confirmation was carried out testing for gram reaction (gram-positive bacilli or coccobacilli), motility (umbrella type), presence of catalase (+) and oxidase (−) and hemolytic activity (+ or −). Colonies complying with these tests were tranferred to TSA slants, incubated for two days at 30°C and further identified using the API *Listeria* (bioMérieux, Lyon, France).

For competitive microorganisms (including lactobacilli) PCA^+-medium was used. Composition (g l^{-1}): plate count agar (PCA, Oxoid CM 325) 13 g, MRSB 13 g and agar (Oxoid L 11) 5 g.

At each sampling three packages were opened aseptically and the total contents were transferred into a stomacher bag. The meat product was diluted (1:2) with PPSS, divided into pieces by hand kneading (30 sec) and subsequently homogenized in a stomacher for 2 min. After 5 min, 15 ml of the homogenate was transferred into sterile tubes and placed in melting ice. Within 1 h, this suspension and appropriate serial dilutions in PPSS were plated onto EHA and PCA^+. Directly after plating, the pH of the samples was determined in the primary dilution. Plates were incubated at 37°C (2 d) for *Listeria* and at 30°C (3 d) for total counts. Growth curves were generated from the experimental data by using the Gompertz equation. The growth rate and lag time were calculated by using a growth-curve fitting program (Zwietering et al. 1990).

Determination of gas atmosphere

In all series concentrations of O_2, CO_2 and N_2 were determined at day 1, after 3 weeks and by the end of shelf life, using a gas chromatograph (Fisons, GC 8000).

Effect of added lactic acid on the pH of luncheon meat and ham

Lactic acid (CCA, Biochem) was added to 100 g meat products (not inoculated) to final concentrations of 0, 0.5. 1.0, 1.5, 2.0 and 2.5 % (w/w). After mixing in a blender (Waring 8010, New Hartford, Connecticut) for 20 sec, the product was placed at 7°C and pH was measured after 1.5, 3 and 24 h.

Production of lactic acid in meat products

After 3, 29 and 39 days of storage, concentration of lactic acid was determined using an enzyme kit (Boehringer lactic acid test combination, 1112821). After mixing 100 g of

meat product in a blender, a 5 g sample was added to 20 ml of 1 M perchloric acid. Then the concentration of lactic acid was determined according to the manufacturer's instructions.

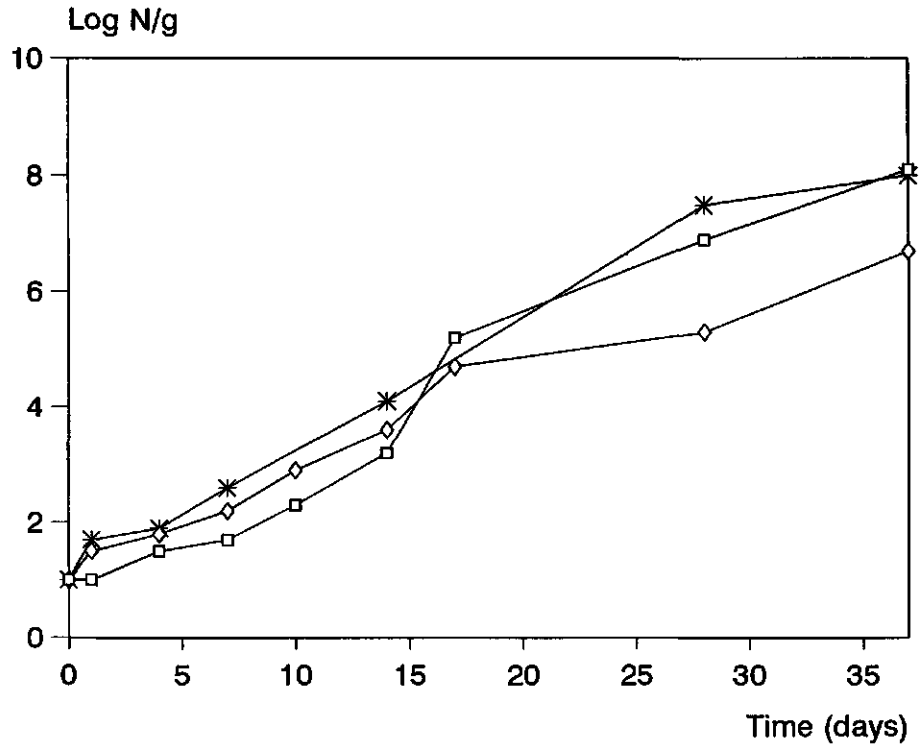

Fig. 1 Growth of *Listeria monocytogenes* on MAP (30% CO_2/70% N_2) cooked ham (◇), luncheon meat (□), and chicken breast (∗) at 7°C

RESULTS

Growth of *L. monocytogenes* and plate counts are presented in the graphs as mean values of three packages. The growth of *L. monocytogenes* on meat products in

30%CO_2/70% N_2 is presented in Fig. 1 and was similar to growth on vacuum packed products (Fig. 2). Within the producer's sell by date, the initial numbers increased up to 10^7-10^8 cfu g^{-1}.

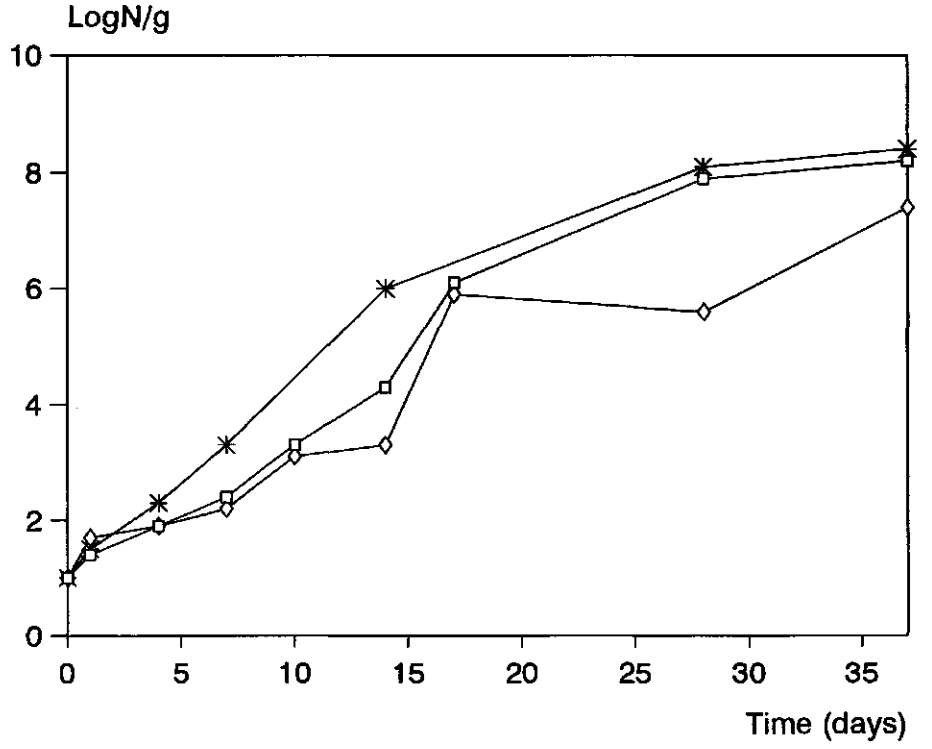

Fig. 2 Growth of *Listeria monocytogenes* on vacuum packaged cooked ham (◇), luncheon meat (□), and chicken breast (∗) at 7°C

Isolation and identification of a number of colonies on PCA^+ indicated that the normal spoilage flora of cooked, vacuum packed or modified atmosphere packed meat products consisted mainly of lactic acid bacteria. *Lb. delbrueckii* subsp. *delbrueckii* and *Lb. curvatus* were the strains most frequently isolated from luncheon meat. Even high numbers of lactic acid bacteria did not affect the growth of *L. monocytogenes* in 30%

CO_2/70% N_2 (Fig. 3) and in the vacuum packed product (Fig. 4).

Leuconostoc mesenteroides subsp. *mesenteroides* and *Lc. lactis* subsp. *lactis* were the predominant spoilage organisms in ham, and *Lb. fermentum* and *Leuc. mesenteroides* subsp. *mesenteroides* in chicken breast. Generation times for lactic acid bacteria in these products were comparable to those in luncheon meat or even shorter. When counts of lactic acid bacteria reached values of about 10^8 cfu g^{-1}, growth of *L. monocytogenes* decreased as is shown for vacuum packed ham in Fig. 4.

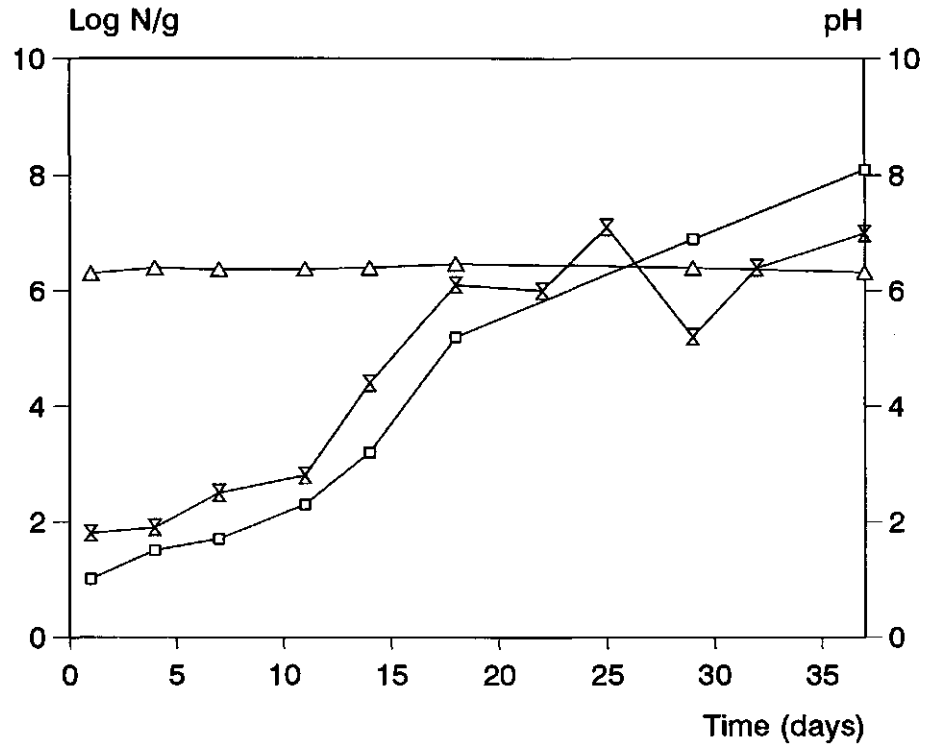

Fig. 3 Growth of *Listeria monocytogenes* (□) and lactic acid bacteria (⧖) on MAP (30% CO_2/70% N_2) luncheon meat, and the effect on pH (△) at 7°C

In spite of the high numbers of lactic acid bacteria in all products, only a slight decrease in pH (about 0.5 unit) was observed. In control samples of ham and chicken breast, both in original packages and in repacked vacuum bags, *L. monocytogenes* was detected.

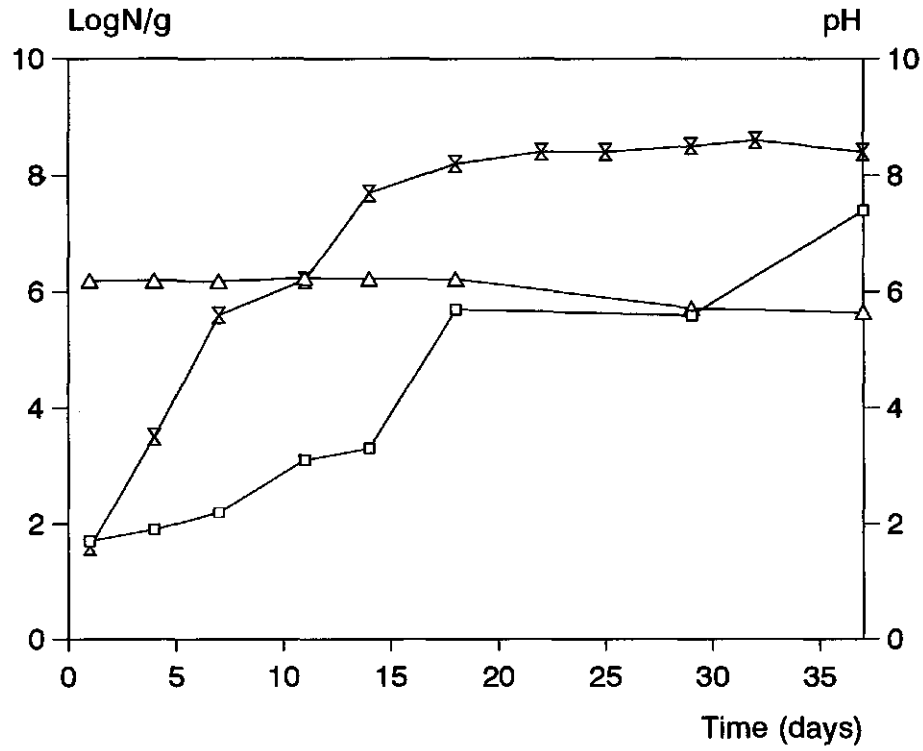

Fig. 4 Growth of *Listeria monocytogenes* (□) and lactic acid bacteria (⧗) on vacuum packaged cooked ham, and the effect on pH (△) at 7°C

Because this species was not detected in all packages, growth curves were somewhat irregular. Fig. 5 shows that these listerias reached numbers of 10^4 cfu g^{-1} at the end of the storage of chicken breast in the original packages. The growth rates on chicken breast for this isolate (μ = 0.57 day^{-1}), the isolate from vacuum packaged chicken breast (μ= 0.55 day^{-1}) and for the test strain (μ = 0.60 day^{-1}) were comparable.

In Table 1 the growth parameters of *L. monocytogenes* in the presence of *Lb. curvatus* are shown. Increasing initial concentrations of lactobacilli decreased the growth rate of *L. monocytogenes* slightly. Since *Lb. curvatus* does not produce antibacterial substances such as bacteriocins, this experiment was repeated with *Lc. lactis*, a nisin producing

Table 1 Parameters of growth of *Listeria monocytogenes* in presence of *Lactobacillus curvatus* on modified gas packaged luncheon meat

Serie	μ^a (day^{-1})	λ^b (days)
Control, repacked	0.62	4.12
Lm[c] + *Lb. curvatus* 10^0 cfu g^{-1}	0.56	2.85
Lm + *Lb. curvatus* 10^1 cfu g^{-1}	0.50	2.72
Lm + *Lb. curvatus* 10^2 cfu g^{-1}	0.49	3.52

[a] μ = growth rate, [b] λ = lag phase, [c] *Lm* = *L. monocytogenes*

strain. Growth rates and lag phases of *L. monocytogenes* were similar to those determined in presence of *Lb. curvatus* (data not shown). No growth of *L. monocytogenes* was observed during the shelf life (6 weeks) of saveloy and (raw) Coburger ham. Initial concentrations of approximately 10^2 cfu g^{-1} decreased to below the detection level (3 cfu g^{-1}).

During storage, only slight differences in gas composition were observed. The O_2 concentration in the original packages decreased from 2 to less than 1%. In the other packages the O_2 concentration varied from 1-3%. Percentages of CO_2 increased from 12 to 28% in the original packages, and from 7 to 23% in repacked bags. In the original packages the N_2 concentration varied from 72 to 86%, which was similar with the concentrations determined in the other packages (76-90%).

Addition of lactic acid to meat products resulted in a decrease in pH, as is shown in Table 2. During the storage of the meat products no increase in lactic acid concentration was detected (data not shown).

DISCUSSION

In this study the growth of *L. monocytogenes* on cooked meat products was investigated. In all experiments meat products were inoculated with diluted overnight cultures of *L.*

monocytogenes, grown for 20-24 h at 30°C. In the production environment contamination with listerias grown at lower temperatures is probably more relevant. However, the growth rates of test strains grown at 7°C and 30°C were similar: μ=0.64 and 0.62 respectively; the growth temperature affected the lag phase only. These results are in

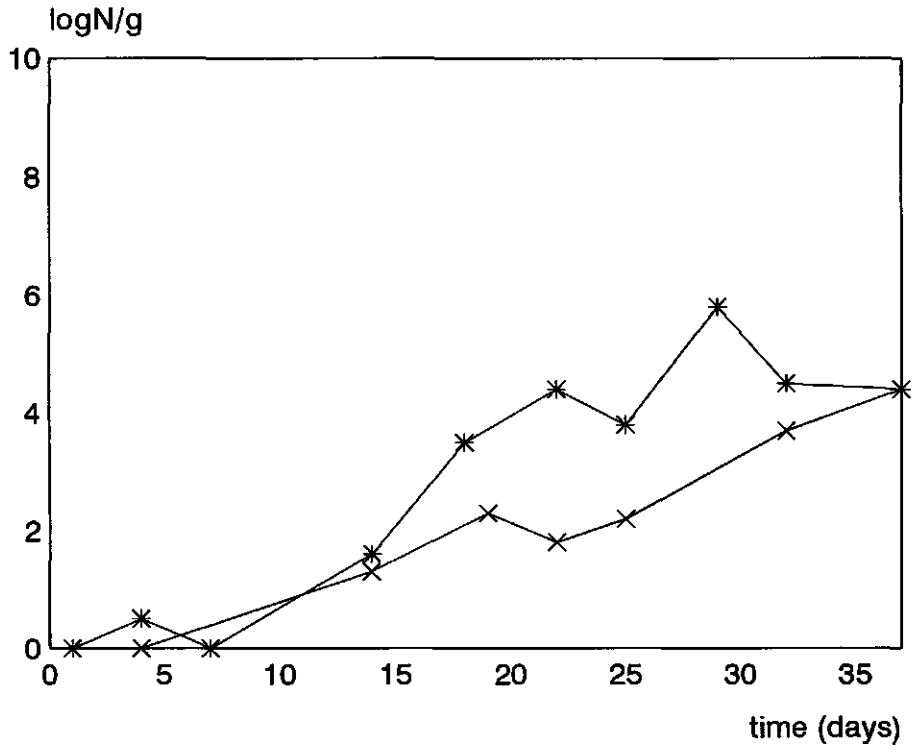

Fig. 5 Growth of *Listeria monocytogenes* on chicken breast in controls (x repackaged in vacuum, * original package) at 7°C

agreement with the findings of Buchanan and Klawitter (1991). Neither the place of inoculation (on the surface or between slices) (data not shown) nor the composition of the gas atmosphere (30% CO_2/70% N_2 or vacuum) (Figs 1-4) influenced growth rate or lag phase. It has been shown previously that packaging of sliced roast beef in a 100%

CO_2 atmosphere extended the shelf life compared to identical product stored under vacuum (Penney et al. 1993). The CO_2 concentrations in the MAP products in this study varied from 7-28%. These concentrations are far too low to inhibit growth of *Listeria*, since growth of *L. monocytogenes* on sliced roast beef (pH 6.1) was even observed in a 100% CO_2 atmosphere at 3°C (Hudson et al. 1994). The effectiveness of MAP increases with decreasing temperatures and O_2 concentrations. But it is unrealistic to maintain temperatures as low as 3°C in the cold chain. The growth of *L. monocytogenes* at 7°C on precooked chicken nuggets in the absence of oxygen (80% CO_2/20% N_2) was similar to the growth we observed in our experiments (Marshall et al. 1991).

Table 2 Influence of added lactic acid on pH of meat products

Lactic acid (%)	pH Luncheon meat	pH Ham
0	6.3	6.1
0.5	5.3	5.2
1.0	4.6	4.7
1.5	4.1	4.3
2.0	3.9	4.1

It is well known that reduced oxygen and increased carbon dioxide concentrations in food packages can increase shelf life via the inhibition of aerobic spoilage bacteria. However, the growth of psychrotrophic *L. monocytogenes* and spoilage organisms of cooked meat products under such environments will not be inhibited as is shown in Figs 1-4. Within the shelf life of the products investigated low initial numbers of *L. monocytogenes* increased up to 10^8 cfu g^{-1}. These results agree with previous work on the fate of *L. monocytogenes* on processed meats (Glass and Doyle 1989, Johnson et al. 1990, McKellar et al. 1994). In our experiments the growth rate of the test strain on chicken breast and luncheon meat was slightly higher than that on ham.

In other studies, *L. monocytogenes* was either incapable of multiplying or increased very slowly on ham. This was contributed to differences in the composition of the products (Grau and Vanderlinde 1992; Farber and Daley 1994), but may also be explained by the use of lower storage temperatures and/or the presence of higher levels of background flora (lactic acid bacteria). It has often been reported that lactic acid

bacteria might inhibit the growth of *Listeria* (McKellar et al. 1994, Schmidt 1995). As can be seen from Figs. 3 and 4 development of *L. monocytogenes* is comparable to that of lactobacilli. This can be explained by the fact that both the inoculation level of *Listeria* (about 10 cfu g^{-1}) and the contamination level of lactic acid bacteria (10-100 cfu g^{-1}) were low. Because of this, mutual interaction between the (micro)colonies formed was unlikely. Increasing the numbers of lactic acid bacteria by a factor 100 resulted in a decrease of the growth of *L. monocytogenes*. However, it still reached numbers of 10^7-10^8 cfu g^{-1}. Other workers have observed similar effects (Hudson et al. 1994). To overcome this outgrowth of *L. monocytogenes*, the use of bacteriocin-producing strains of lactic acid bacteria has been proposed (Berry et al. 1991, Foegeding et al. 1992). In our experiments we could not demonstrate a stronger inhibitory effect by the use of a nisin-producing *Lc. lactis*. Growth rates of *L. monocytogenes* were similar to those of *Lb. curvatus* (Table 1). In all probability effective concentrations of nisin are only present when high levels of lactococci are reached (in the late log phase). In an experiment where nisin (Sigma N 5764) was added in a concentration of 25 mg kg^{-1} to luncheon meat, there was an initial decrease in numbers of *L. monocytogenes*. After storage for one week at 7°C, the levels were about two log units lower compared to the controls, but at the end of the storage time (after 6 weeks) counts in both products exceeded 10^7 cfu g^{-1} (data not shown). This phenomenon is due to the rapidly decreasing activity of nisin on meat surfaces (Chung et al. 1989, Fang and Lin 1994).

Adding 0.5% lactic acid to meat products resulted in a decrease in pH of about one unit. At the end of the storage of cooked meat products the decrease in pH was approximately 0.5 unit. The amount of lactic acid necessary for this reduction could not be detected due to the detection limit of the test used.

The decrease in numbers of *L. monocytogenes* on saveloy and Coburger ham can be explained by the low pH of these products (saveloy 4.3−4.5 and raw ham 5.5−5.7). This agrees with results obtained by others (Sabel et al. 1991, Farber et al. 1993).

Since spoilage may not be evident in MAP foods, consumers could judge such products as safe even in the presence of high numbers of pathogens. Processors should be aware of this and take measures to prevent recontamination of the products, particularly with pathogens. Because such steps will never fully guarantee the absence of a single pathogen, the shelf life of products supporting the growth of *L. monocytogenes* and/or other pathogens should be restricted.

Since recontamination of cooked meat products with *L. monocytogenes* is usually limited, only few (micro)colonies will be formed on the meat during storage. Therefore, it is recommended to examine large portions (e.g. whole packages) to increase the chance to detect *L. monocytogenes*.

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10

EFFECT OF EXOGENOUS PROLINE, BETAINE AND CARNITINE ON GROWTH OF *LISTERIA MONOCYTOGENES* IN A MINIMAL MEDIUM

RR Beumer, MC te Giffel, LJ Cox, FM Rombouts and T Abee

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SUMMARY

Three *Listeria monocytogenes* strains isolated from food or food processing environment were used to assess their response to salinity in a chemically defined minimal medium. Growth in a minimal medium containing five essential amino acids and glucose as a carbon- and energy source was comparable to growth in a rich medium (brain heart infusion broth). In the absence and presence of NaCl (3%) final cell numbers reached in minimal medium were 10^9 and 10^7 cfu ml^{-1}, respectively. Growth in the latter condition could not be detected by spectrophotometry measuring absorbance at 660 nm. Apparently this technique is not suitable for these experiments since the detection level is $>10^7$ cfu ml^{-1}.

Exogenously added proline (10 mM), trimethylglycine (betaine, 1 mM) and ßhydroxy-γ-N trimethyl aminobutyrate (carnitine, 1 mM) significantly stimulated growth under osmotic stress conditions in minimal medium both at 37 and 10°C.

Betaine and carnitine are present in foods derived from plant and animal origin, respectively. These compounds can therefore contribute significantly to growth of *L. monocytogenes* in various foods at high osmolarities.

10

INTRODUCTION

Listeria monocytogenes has been recognized as an important foodborne pathogen in recent years. This organism is particularly problematic for the food industry because it is widespread in the environment (Farber and Peterkin 1991). *L. monocytogenes* is able to grow over a wide range of temperatures (1 to 45°C), pH values (5 to 9), and osmotic pressures (NaCl concentrations up to 10%). It is also facultatively anaerobic (Seeliger and Jones 1986). Thus, many of the traditional preservative systems used in foods, alone or in combination, have little effect on the multiplication of the organism. The tolerance of the organism to osmotic stress suggests that it can adapt to the osmotic environment.

Osmoregulation has been studied most extensively in the Gram-negative bacteria *Escherichia coli* and *Salmonella typhimurium*. Adaptation of these bacteria to high extracellular solute concentrations is generally accompanied by the intracellular accumulation of potassium ions and organic compounds such as amino acids, imino acids, quaternary ammonium compounds, and carbohydrates (i.e. trehalose) (Csonka 1989).

The physiological responses of Gram-positive bacteria to changes in external osmotic potential has received less attention. *Streptomyces* species show increased synthesis of proline, glutamine, and alanine resulting in high intracellular concentrations of these compounds, when the organisms are grown in the presence of increased salt concentrations (Killham and Firestone 1984). An important role has been observed for the osmoprotectants proline and N,N,N-trimethylglycine (betaine) [$(CH_3)_3$-N^+-CH_2-COO^-] in the osmoregulation of *Staphylococcus aureus* and *Lactobacillus acidophilus* (Bae and Miller 1992, Jewell and Kashket 1991, Pourkomailian and Booth 1992). Recently, Patchett et al. (1992) described the effect of sodium chloride on the intracellular solute pools of *L. monocytogenes* in a rich undefined broth. *L. monocytogenes* cells grown in the presence of 7.5% NaCl contained higher concentrations of K^+, betaine, glycine, alanine, and proline than cells grown in the absence of NaCl. However, it is not clear to what extent uptake and/or synthesis of these osmolytes contributes to osmoprotection in *L. monocytogenes*.

Betaine is a trimethyl amino acid and occurs in high concentrations in sugar beets and other food products of plant origin. A trimethyl amino acid that is structurally related to betaine and might act as an osmoprotectant is ß-hydroxy-γ-N-trimethyl aminobutyrate (carnitine) [$(CH_3)_3$-N^+-CH_2-CHOH-CH_2-COO^-]. Materials of plant origin tend to be low

in carnitine, while materials of animal origin are particularly rich sources of this compound (Mitchell 1978). Carnitine has also been added to some foods, such as infant formulas, because of its vitamin-like activity (Augustin and Scarbrough 1989).

To understand the ability of *L. monocytogenes* to grow and survive in widely diverse environments, it is necessary to determine the nutritional requirements of this organism in a chemically defined minimal medium (MM) and how these requirements are affected under various stress conditions. In this study a MM that was based on a medium described by Premaratne et al. (1991) containing five essential amino acids was used to determine the influence of incubation temperature, salt concentration and various osmoprotectants on the growth of *L. monocytogenes*.

MATERIALS AND METHODS

Medium composition

A chemically defined MM based on a medium described previously (Premaratne et al. 1991) was used. This medium contained (per liter): KH_2PO_4 6.56 g, Na_2HPO_4 30.96 g, $MgSO_4$ 0.41 g, ferric citrate 0.088 g, glucose 10.0 g, L-leucine 0.1 g, L-isoleucine 0.1 g, L-valine 0.1 g, L-methionine 0.1 g, L-cysteine 0.1 g, L- glutamine 0.6 g, riboflavine 0.5 mg, thiamine 1.0 mg, biotin 0.5 mg, and thioctic acid 0.005 mg.

Trimethylglycine (betaine, Sigma B2629), L-proline (Sigma P0380) and ß-hydroxy-γ-N-trimethyl aminobutyrate (DL-carnitine, Sigma C9500) were each added to MM as filter sterilized solutions to a final concentration of 1 mM, unless indicated otherwise.

Strains

Three *L. monocytogenes* strains obtained from the culture collection of NESTEC (Switzerland) were used. Strains 1 (serovar 1/2b), 106 (serovar 1/2a), and 311 (serovar 3a) were isolated from food or food-processing environments. The strains were stored at - 80°C in brain heart infusion broth (BHIB, Gibco 15200680) containing 20% glycerol.

Inoculum preparation

To prepare each initial inoculum, 0.1 ml of an overnight culture was transferred into 10 ml of BHIB and was incubated at 37°C for 18 h. Cells were harvested by centrifuging at 2.600 g for 15 min. The pellets were washed twice and resuspended in 10 ml MM.

Growth of *L. monocytogenes* in MM and BHIB

Erlenmeyer flasks containing 100 ml of MM or BHIB were inoculated with about 10 cells ml^{-1} and incubated statically at various temperatures. The inoculum level was checked by surface streaking on tryptone soya agar (TSA, Oxoid CM 131). Samples were removed regularly and plated (after dilution) on TSA. The TSA plates were incubated at 30°C for 2 days. Growth curves were generated from the experimental data using the Gompertz equation. The growth rate and lag time were calculated using a growth curve-fitting program (Zwietering et al. 1990).

Growth of *L. monocytogenes* in MM under osmotic stress conditions

Optical density measurements

Tubes containing 10 ml of MM or BHIB containing various salt concentrations with or without additional osmoprotectants (see above) were inoculated (10^7 cfu ml^{-1}) and incubated statically at 37°C for 30 h. Growth was measured spectrophotometrically by determining the optical density at 660 nm (OD_{660}) (Beckman Instruments BV, Mijdrecht, Holland).

Plate count experiments

Erlenmeyer flasks containing 100 ml of MM were inoculated with about 10 cells ml^{-1} and incubated statically at 37 or 10°C. Cells were grown at a high osmotic strength (3% NaCl) in the absence and presence of betaine (1 mM) or carnitine (1 mM). The inoculum level was checked by surface streaking on TSA. Samples were removed regularly, plated (after dilution) on TSA plates, and incubated at 30°C for 2 days.

RESULTS

Growth of *L. monocytogenes* in MM and in BHIB

An analysis of the growth requirements revealed that all three *L. monocytogenes* strains were able to grow in a minimal glucose medium containing the following five essential amino acids: L-leucine, L-isoleucine, L-valine, L-methionine, and L-cysteine (data not shown). Table 1 shows the lag time and growth rate for strain 311 as calculated with the curve-fitting program. Similar results were obtained for the other strains (data not shown). Although there were small differences in growth rate and lag time, growth in MM was comparable to growth in BHIB. This indicates that all of the factors essential for growth were present in MM.

Table 1 Growth of *Listeria monocytogenes* 311 in MM and BHI at different temperatures[a]

Temp. (°C)	Lag time (h) in:		Growth rate (h^{-1}) in:	
	MM	BHI	MM	BHI
37	3.0 ± 1.3	3.1 ± 0.8	0.97 ± 0.19	1.29 ± 0.17
30	3.8 ± 1.9	3.9 ± 1.2	1.20 ± 0.40	1.60 ± 0.40
20	9.6 ± 2.4	6.9 ± 2.5	0.51 ± 0.07	0.59 ± 0.09
10	55.2 ± 8.2	26.0 ± 9.9	0.11 ± 0.009	0.13 ± 0.03
4	499.0 ± 16	ND[b]	0.03 ± 0.003	ND

[a] Experiments were repeated three times, [b] ND = not determined

Growth of *L. monocytogenes* in MM supplemented with different salt concentrations

Fig. 1 shows that *L. monocytogenes* 311 was able to grow in the presence of higher NaCl concentrations in a rich medium than in MM. The same effects were observed with the other strains (data not shown). Addition of up to 5% NaCl in BHIB had very little effect, although there were small differences between strains (data not shown). In MM increasing salt concentrations led to a decrease in the final optical density, indicating that growth was severely reduced. In MM supplemented with 5% NaCl the OD_{660nm} reached was only 3% of the OD_{660} reached after growth in MM in absence of NaCl. Similar results were obtained for KCl, although KCl was less inhibitory to growth than NaCl (data not shown). Apparently, compounds present in BHIB allow *L. monocytogenes* to grow at osmotic strengths that are otherwise inhibitory.

Growth of *L. monocytogenes* in MM at a high osmotic strength as influenced by proline, betaine and carnitine

When betaine was added to a final concentration of 1 mM, it stimulated the growth of *L. monocytogenes* in MM at high osmolarities. After 30 h of incubation, the OD_{660} values reached in MM supplemented with 3% NaCl in the absence or presence of betaine were 0.03 and 0.50, respectively (Fig. 2). *L. monocytogenes* was able to grow in the presence of NaCl concentrations up to 5% in MM in the presence of betaine. There was no difference between the results obtained when betaine was added at the beginning of the experiment (zero time) and the results obtained when betaine was added during the exponential growth phase (after 10 h). The addition of proline (1 mM) had no effect on *L. monocytogenes* growth in MM in the presence of different NaCl concen-

trations (data not shown). However, when proline was added to a final concentration of 10 mM, significant stimulation of growth was observed (Fig. 2). Betaine was apparently more effective than proline, since higher final OD_{660} values were reached. When the osmotic strength was varied with KCl, similar results were obtained, and the osmotic

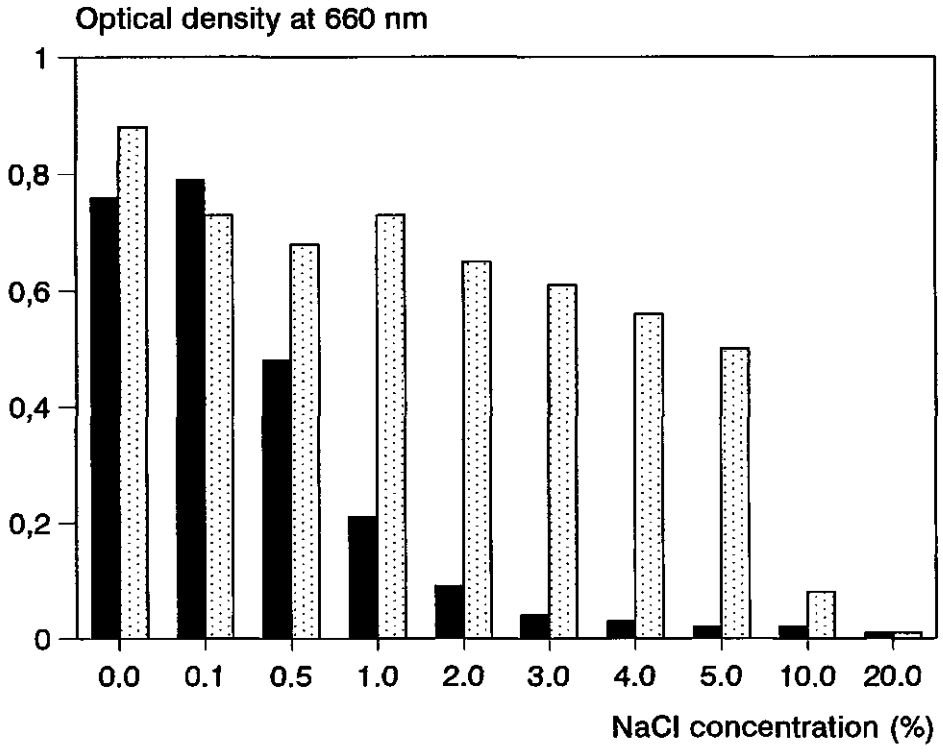

Fig. 1 Growth of *Listeria monocytogenes* 311 in MM (solid bars) and BHIB (stippled bars) in the presence of NaCl at 37°C

tolerance of *L. monocytogenes* strains MM increased when 1 mM betaine or 10 mM proline was added. In contrast, betaine or proline did not have any effect on *L. monocytogenes* growth in BHIB with increasing NaCl concentrations (data not shown). In the absence of osmoprotectants, no increase in OD_{660} was observed, suggesting that

L. monocytogenes was not able to grow in MM with 3% NaCl (Fig. 3). When carnitine (1 mM) or betaine (1 mM) was added to MM containing 3% NaCl, growth was significantly stimulated. The final OD_{660} reached was approximately the same as the OD_{660} reached in MM in the absence of NaCl. No difference in growth stimulation by DL-carnitine or L-carnitine (Sigma C7518) were observed (data not shown). These results

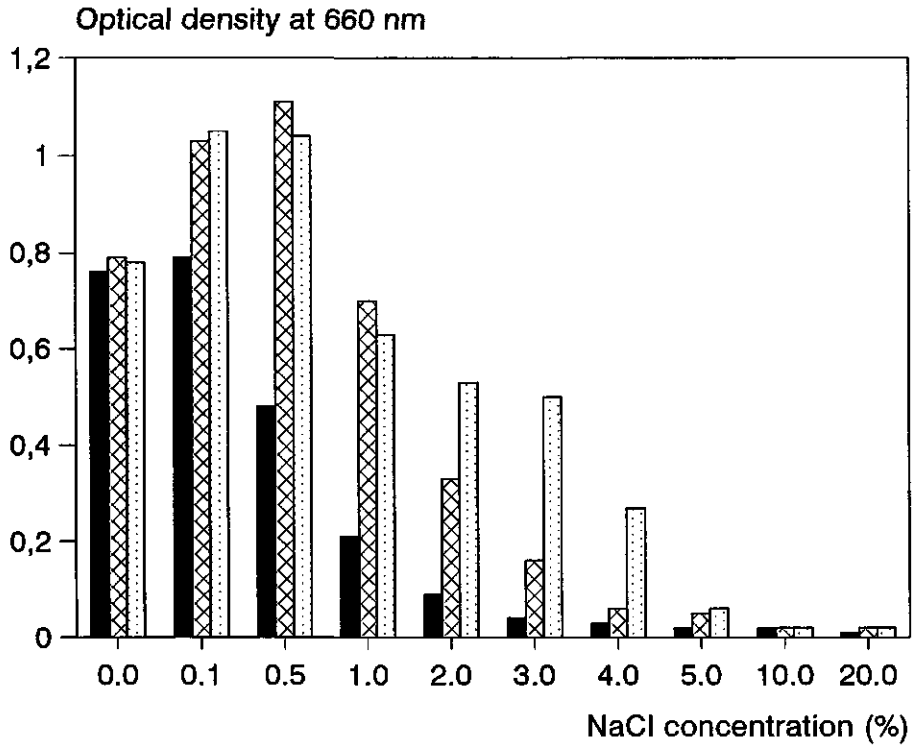

Fig. 2 Growth of *Listeria monocytogenes* 311 in MM (solid bars) as influenced by 10 mM proline (chequered bars) or 1 mM betaine (stippled bars) in the presence of NaCl at 37°C

indicate that both carnitine and betaine function very effectively as osmoprotectants in osmotically stressed *L. monocytogenes* cells.

In order to analyze the growth of microorganisms by spectrophotometry, high numbers of microorganisms are needed since the detection level of this technique is 10^6-10^7 cfu ml^{-1}.However, initial contamination levels in food are generally much lower than this. Therefore, we investigated the effects of salt and osmoprotectants on the growth of low numbers of microorganisms in MM. Inoculum levels of 10 cfu ml^{-1} were used, and growth was monitored under different stress conditions. The levels reached in the stationairy phase for growth of *L. monocytogenes* in MM containing 3% NaCl and the osmoprotectant betaine (1 mM) or carnitine (1 mM) were comparable to the levels reached in MM in absence of NaCl (about 10^9 cfu ml^{-1}) (Fig. 4). The growth rates in

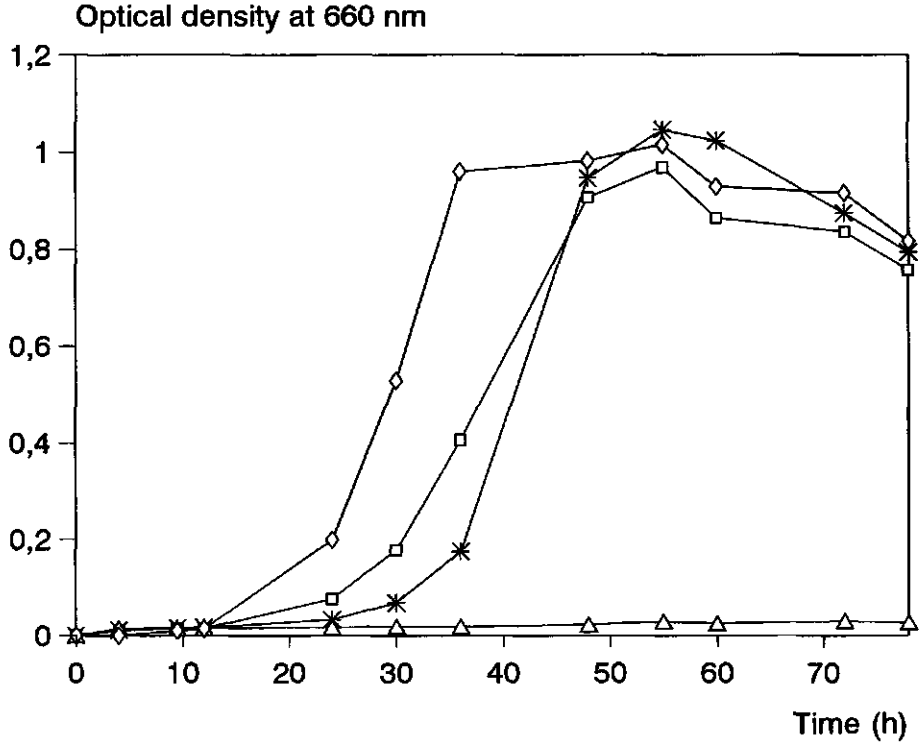

Fig. 3 Growth of *Listeria monocytogenes* 106 in MM (◇), in MM supplemented with 3% NaCl (△), and in MM with 3% NaCl and 1 mM betaine (∗) or 1 mM carnitine (□) at 37°C, as determined by OD_{660}

MM supplemented with 3% NaCl and betaine and in MM supplemented with 3% NaCl and carnitine were 0.69 h^{-1} and 0.59 h^{-1}, respectively.

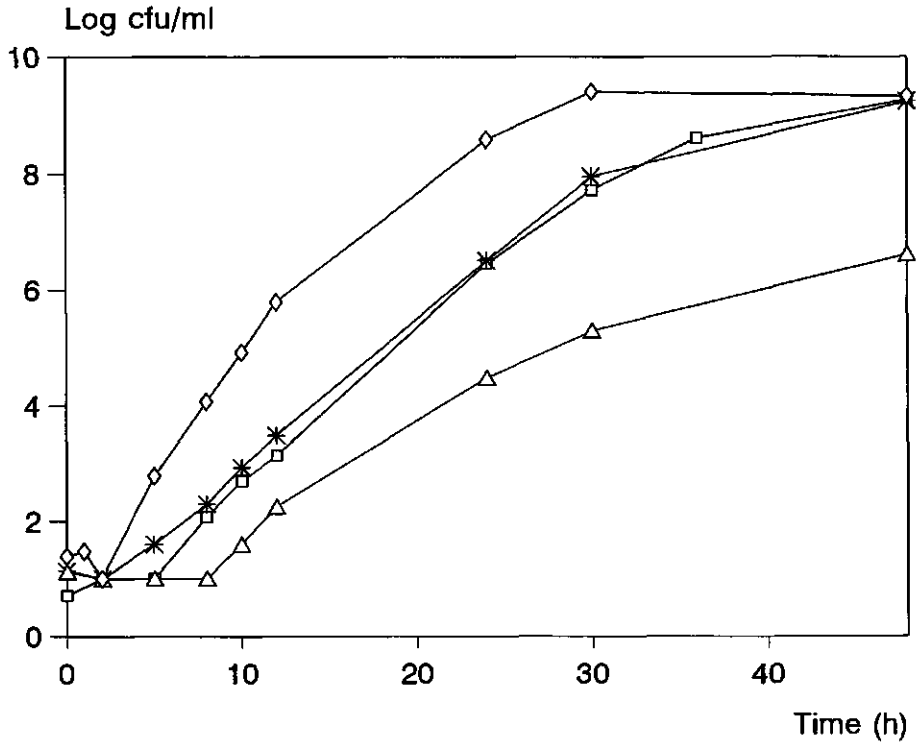

Fig. 4 Growth of *Listeria monocytogenes* 106 in MM (◇), in MM supplemented with 3% NaCl (△), and in MM with 3% NaCl and 1 mM betaine (∗) or 1 mM carnitine (□) at 37°C, as determined by plate counts

Strikingly, growth was also observed in the absence of osmoprotectants in MM with 3% NaCl (growth rate, 0.53 h^{-1}). However, the maximum level reached in the stationary phase was 10^6-10^7 cfu ml^{-1} (Fig. 4). This indicates that the presence of osmoprotectants mainly affected the ultimate cell density.

Growth of *L. monocytogenes* in MM at a high osmotic strength and a low temperature

Cells were grown at 10°C since this might give more realistic information concerning the growth of *L. monocytogenes* in refrigerated food. Increasing the NaCl concentration inhibited *L. monocytogenes* growth at 10°C, and the pattern was similar to the pattern observed for growth at 37°C. Externally added betaine (1 mM) or carnitine (1 mM) reduced the lag time and resulted in increased *L. monocytogenes* growth rates in media whose osmotic strength was otherwise inhibitory (Fig. 5).

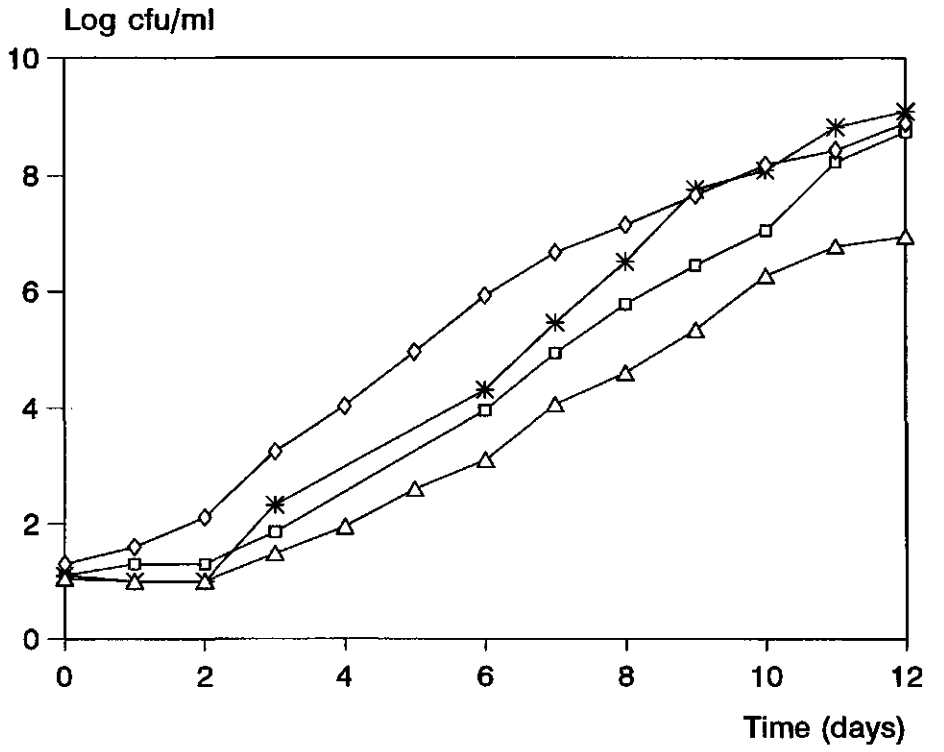

Fig. 5 Growth of *Listeria monocytogenes* 1 at 10°C in MM (◇), in MM supplemented with 3% NaCl (△), and in MM with 3% NaCl and 1 mM betaine (*) or 1 mM carnitine (□)

The final cell numbers reached in the stationary phase in the presence of betaine or carnitine were comparable to those attained during growth in MM in the absence of salt (10^9 cfu ml^{-1}). Growth was again observed in MM supplemented with 3% NaCl in the absence of osmoprotectants, but this resulted in a final population density that was 2 log units lower (Fig. 5).

DISCUSSION

In this study three *L. monocytogenes* strains isolated from food or food-processing environments were used to examine *L. monocytogenes* growth in a chemically defined MM. Growth of *L. monocytogenes* in a minimal glucose medium depends on the presence of five essential amino acids. *L. monocytogenes* is able to grow in many food products because of the general availablity of these (essential) amino acids in food. Food products such as fresh meat contain 0.1 to 0.3% free amino acids (fresh weight basis), and all amino acids are detectable in low amounts (Belitz and Grosch 1986).

Growth of *L. monocytogenes* in MM was comparable to growth in BHIB. The growth rate in BHIB decreased by a factor of 10 when the temperature decreased from 30 to 10°C. In MM the growth rate decreased by a factor of 12. This indicates that at lower temperatures the medium composition becomes more important. The uptake of protective factors, which are present in BHIB but not in MM, could play an important role in the growth of *L. monocytogenes* strains at low temperatures. The nature of such factors remains to be elucidated. Another possible explanation is that at low temperatures energy requirements for maintenance are higher, resulting in better growth in rich media than in MM.

L. monocytogenes can grow in the presence of a wide range of salt concentrations in complex media. High concentrations of NaCl decreased the growth rate and specific growth yield more than high concentrations of KCl. This effect has also been reported for *Streptomyces* species (Killham and Firestone 1984). The optical density experiment showed that the final OD_{660} reached in MM was lower than the final OD_{660} reached in BHIB. This was most likely due to the presence of osmoprotective factors in BHIB, since addition of proline (10 mM) or betaine (1 mM) did not further stimulate growth of *L. monocytogenes* in this medium at a high osmotic strength.

It has been well documented that exogenously added proline and betaine accumulate as compatible solutes in Gram-positive microorganisms (Graham and Wilkinson 1992, Townsend and Wilkinson 1992). In this study we found that exogenous proline, betaine

and carnitine stimulated the growth of *L. monocytogenes* in the presence of high salt concentrations. Under these conditions proline at a concentration of 10 mM stimulated *L. monocytogenes* growth. Patchett et al. (1992) concluded that proline could not function as an osmoprotectant for *L. monocytogenes* during growth at high osmotic strengths. The results of these authors could be explained by the low concentration of proline (1 mM) that they used in their experiments. Low concentrations of proline (< 1 mM) increase the osmotic tolerance of Gram-negative organisms. For Gram-positive organisms it has been reported that higher concentrations (up to 50 mM) are needed to produce a significant osmoprotective effect (Jewell and Kashket 1991). Therefore, proline can contribute significantly to the osmotic tolerance of *L. monocytogenes* in media containing relatively high proline concentrations in the absence of betaine, or its precursors or betaine analogues. In food, proline plays a minor role in osmoprotection since free proline is available only in small amounts (it is present mainly in bound form in proteins). However, it is conceivable that the liberation of proline from proteins by the proteases of other bacteria in foods could result in the availability of this amino acid to *L. monocytogenes*, resulting in growth stimulation at high osmolarities. Betaine and carnitine stimulated growth of *L. monocytogenes* in a high-osmotic-strength medium at concentrations as low as 1 mM at both high and low temperatures. Betaine is mainly present in foods of plant origin. Choline (a precursor of betaine) and carnitine are present in muscle tissue at concentrations of 0.02 to 0.06 and 0.05 to 0.2% (on a fresh weight basis), respectively. These levels, ranging from 1 to 10 mM, are comparable to the concentrations used in our experiments. γ-Butyrobetaine, a carnitine precursor, is known to act as an osmoprotectant for *Klebsiella pneumoniae* in high-osmotic-strength media (Le Rudulier et al. 1984). Betaine and carnitine were equally effective in stimulating *L. monocytogenes* growth under high-osmotic-strength conditions. Whether carnitine is accumulated, metabolized or converted to another compound which functions as the actual osmoprotectant remains to be elucidated.

Experiments in which low inoculum levels were used showed that *L. monocytogenes* was able to grow in MM in the presence of high NaCl concentrations (Fig. 3 and 4). Addition of NaCl resulted in increased lag times and decreased growth rates, and the final cell numbers reached in the stationary phase decreased to 10^6 -10^7 cfu ml^{-1}. This suggests that additional energy, most likely for the synthesis of osmolytes, is used to allow growth at high osmolarities. In general, cell numbers greater than 10^7 cfu ml^{-1} are needed before growth can be detected by spectrophotometry; this is why this method

cannot be applied when low inoculum levels are used. When a high inoculum level (10^7 cfu ml^{-1}) was used, no growth of *L. monocytogenes* in MM supplemented with high NaCl concentrations was observed, as determined by plate count experiments and by spectrophotometry (OD_{660}), indicating that the composition of MM was such that it did not allow growth of large cell numbers under these conditions. Addition of osmoprotectants (10 mM proline, 1 mM betaine, and 1 mM carnitine) significantly stimulated growth, and high cell numbers could be reached.

In conclusion, we found that *L. monocytogenes* can use various exogenously supplied osmoprotectants that allow growth under osmotic stress conditions. Betaine is currently recognized as the most widespread compatible solute (Csonska 1989). However, in food and food systems, especially those of animal origin, such as meat and dairy products, carnitine may function as the main compatible solute that allows *L. monocytogenes* to grow at high osmolarities.

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11

LISTERIA SPP. IN DOMESTIC ENVIRONMENTS

RR Beumer, MC te Giffel, E Spoorenberg and FM Rombouts

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SUMMARY

Using a direct isolation method *Listeria* spp. were detected in 101 (47.4%) of the 213 houses investigated. In 45 (21.1%) *L. monocytogenes* was present. *Listeria* occurred at all sampling sites. Dish-cloths (37%) and surface samples round the drain in the bathroom (27.2%) were most frequently contaminated. Highest numbers (ca 10^5 cfu/object) were found in dish-cloths and washing-up brushes. For the kitchen sink samples, the refrigerator vegetable compartment samples and tooth brushes lower levels (up to 10^3 cfu/object) were observed. In total, 132 *Listeria* spp. were isolated from 871 samples. *L. innocua* (53%) and *L. monocytogenes* (41%) were the predominant species in the positive samples. Other *Listeria* spp. were found in only 6% of the positive samples.

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INTRODUCTION

Listeria monocytogenes has been isolated from an extensive range of raw and processed food products including pâté and other cooked meat products (Gilbert et al. 1993), soft cheeses (Pini and Gilbert 1988), (smoked) fish products (Dillon and Patel 1992, Eklund et al. 1995) and vegetables (Breer and Baumgartner 1992). When this pathogen is isolated from processed foods, it is usually not a result of inadequate heat processing, but rather post-process contamination of the product. In most cases, *L. monocytogenes* can be isolated from the environment of food plants which have produced *Listeria* contaminated product (Ryser and Marth 1991, Jacquet et al. 1993). In food factories, listerias were found in drains, floors, standing water, residues and food contact surfaces in descending order of frequency. In dry culinary food units no samples were found to be contaminated, which indicates that dry conditions and the restriction of food residues contribute to the control of these organisms (Cox et al. 1989).

Although various food products have been implicated in outbreaks of listeriosis, the source and route of infection are usually unknown. The investigation of a case of listeriosis, whether sporadic or in the context of an epidemic, is often hampered both by the limitations of traditional typing techniques and by the occurrence of strains unrelated to the outbreak strain in implicated food products and in the environment (Kerr et al. 1995). This can be contributed to the isolation methods used. During enrichment the presence of the strain isolated from patients may be masked by faster growth of other *Listeria* spp. present in the sample (Due and Schaffner 1993; Petran and Swanson 1993; Curiale and Lewus 1994) (Chapter 3). It cannot be ruled out that unknown sources of *L. monocytogenes* may also be responsible for illness. Although presence of *Listeria* spp. in household environments has been demonstrated (Cox et al. 1989), these environments have not frequently been included in epidemiological studies. Particularly wet places may be niches which favour survival and growth of listerias. This suggests that humans may be regularly exposed to these organisms from sources other than contaminated food.

In this study the occurrence of *Listeria* spp. was investigated in 213 households in The Netherlands. Samples were taken from six domestic environments such as dish-cloths, washing-up brushes, tooth-brushes, the bathroom (shower), the kitchen sink and the vegetable compartment of the refrigerator. Since low numbers of listerias probably play a minor role in contamination and infection, a direct (selective) plating procedure was used.

MATERIAL AND METHODS

Growth experiments

Appropriate dilutions in peptone physiological saline solution [PPSS; composition: NaCl (8.5 g l^{-1}) and neutralized bacteriological peptone (Oxoid L34, 1 g l^{-1})] of an overnight culture 20 h at 37°C) of *L. monocytogenes* (isolated from a dish-cloth) in brain heart infusion broth (BHIB, Difco 0037-01-6) were used to inoculate dish-cloths, washing-up brushes and tooth-brushes (ca 10^3 cells/object). The inoculum level was checked by plating on tryptone soya agar (TSA, Oxoid CM 131). To check the recovery, the survival and the growth of *Listeria*, objects were investigated half an hour after inoculation and after 24 h incubation at room temperature (without any covering), according to the procedure described under enumeration of *Listeria* spp.

Sampling sites and methods of sampling

In 213 (at random selected) households, surface samples (20 cm^2) were taken in bathrooms, from the kitchen sink (at both sites around the drain) and from the bottom of the vegetable compartment in refrigerators. For each area two swabs (Falcon, 36/2096) were used. With a swab, moistened in PPSS with 0.1% Tween 80 (Merck 822187) (PPSST), the surface was sampled with a rotary movement, at first in north/south direction, followed by an east/west movement. Then the surface was sampled in the same way with a dry swab. Both swabs were placed in a tube with 3 ml PPSST. Moreover, in all households we asked for dish-cloths, washing-up brushes and tooth-brushes, which were packed in sterile stomacher bags. All samples were transported to the laboratory in melting ice, in insulated cool boxes, and investigated within two hours. Type of house, number of residents and time and day of sampling were listed.

Enumeration of *Listeria* spp.

Tubes with swabs were mixed thoroughly by Vortex mixing (1 minute). One ml PPSST was distributed on three enhanced hemolysis agar plates (EHA, composition and preparation as in chapter 4) and from a 10-fold dilution in PPSS, 0.1 ml was transferred (in duplicate) to EHA plates.

PPSST was added to dish-cloths and washing-up brushes (50 ml) and to tooth brushes (15 ml). Stomacher bags were mixed vigorously by shaking (1 minute). From each sample one ml was distributed on three EHA plates and 0.1 ml from a 10-fold dilution in PPSS was transferred (in duplicate) to EHA plates.

EHA plates were incubated for two days at 37°C. All *Listeria* spp. appear to be fluorescent under UV light (366 nm), in addition *L. monocytogenes* shows hemolysis. Typical *Listeria* colonies were purified by streaking on a non-selective medium (TSA) and incubated for two days at 30°C. With colonies demonstrating a characteristic blue colour under Henry illumination, confirmation was carried out testing for Gram reaction (Grampositive bacilli or coccobacilli), motility (umbrella type), presence of catalase (+) and oxidase (−) (Lovett 1988) and hemolytic activity (+ or −) (Dominquez et al. 1986). Colonies complying with these tests were tranferred to TSA slants, incubated for two days at 30°C and further identified using the API *Listeria* (bioMérieux, Lyon, France) according to the manufacturer's instructions (see Chapter 6).

RESULTS

To determine whether it was possible to recover listerias from the samples according to the proposed procedure, this was checked first using artificially contaminated samples. With the methods used we were able to isolate the listerias (recovery 75-120%). After one day at room temperature (20°C) a slight increase in numbers was observed, particularly in used objects (Table 1).

Table 1 Recovery and growth of *Listeria* spp. from/on artificially contaminated dish-cloths, washing-up brushes and tooth-brushes stored at room temperature (22°C)

Sample (#)	Added cells/object	Recovery (%)	
		after 30 min	after 24 h
tooth-brushes			
new (3)	1500	75 ± 1.6[a]	77 ± 7
used (3)	1500	76 ± 15	108 ± 26
washing-up brushes			
new (3)	3000	120 ± 2	144 ± 23
used (3)	3000	117 ± 21	162 ± 14
dish-cloths			
new (3)	3000	94 ± 16	103 ± 19
used (3)	3000	80 ± 15	156 ± 15

[a] mean + standard deviation of 3 experiments

In Table 2 the occurrence of *Listeria* spp. at various sampling sites is presented. From 871 samples 132 listerias were isolated. *L. innocua* (53%) and *L. monocytogenes* (41%) were the predominant species in the positive samples. Other *Listeria* spp. were found in only 6% of the positive samples.

The mean contamination levels for *L. innocua* and *L. monocytogenes* (LogN/object) are given in Fig. 1. Highest counts were found on dish-cloths, washing-up brushes and tooth-brushes (10^4-10^5 cfu/object). Lower numbers (10^2-10^3 cfu/object) were detected in the bathroom, the kitchen sink and in the vegetable compartment. Mean contamination levels for the other *Listeria* spp. were comparable with those observed for *L. innocua* and *L. monocytogenes* (data not shown).

For samples from the bathroom, washing-up brushes and dish-cloths a relative frequency distibution is given in Fig. 2a,b,c. In 70% of the positive samples from the bathroom and in all samples from washing-up brushes and dish-cloths numbers of *L.*

Table 2 Occurrence of *Listeria* spp. in domestic environments

Type (and #) of samples	***Listeria* (#, %)**[a]	**Isolated *Listeria* spp.**		
		L. innocua	***L. monocytogenes***	**Other spp.**
dish-cloth (108)	40 (37)	20	18	1 (*L. seeligeri*) 1 (*L. welshimeri*)
washing-up brush (96)	12 (12.5)	6	6	
kitchen sink[b] (210)	12 (5.7)	8	3	1 (*L. grayi*)
refrigerator[b,c] (204)	8 (3.9)	3	5	
bathroom[b] (206)	56 (27.2)	30	21	1 (*L. ivanovii*) 1 (*L. grayi*) 2 (*L. seeligeri*) 1 (*L. welshimeri*)
tooth-brush (47)	4 (8.5)	3	1	
Total (871)	132 (15.2)	70	54	8

[a] number (and percentage) of samples containing *Listeria*, [b] swab samples (20 cm^2), [c] vegetable compartment

monocytogenes were higher than 10^2 cfu/object. In about 80% of the positive dish-cloths and washing-up brushes, this pathogen was present in numbers $>10^3$ cfu/object.

Various types of accommodation have been investigated, including detached villas, terrace houses, apartments and student apartments (n=213). The level of contamination of the samples was similar in the different types of housing. In 101 of the 213 houses examined (47.4%) *Listeria* spp. were detected. In 74 of the 101 houses (73%) *Listeria* was isolated from only one of the six samples investigated. In 23 houses (23%) listerias

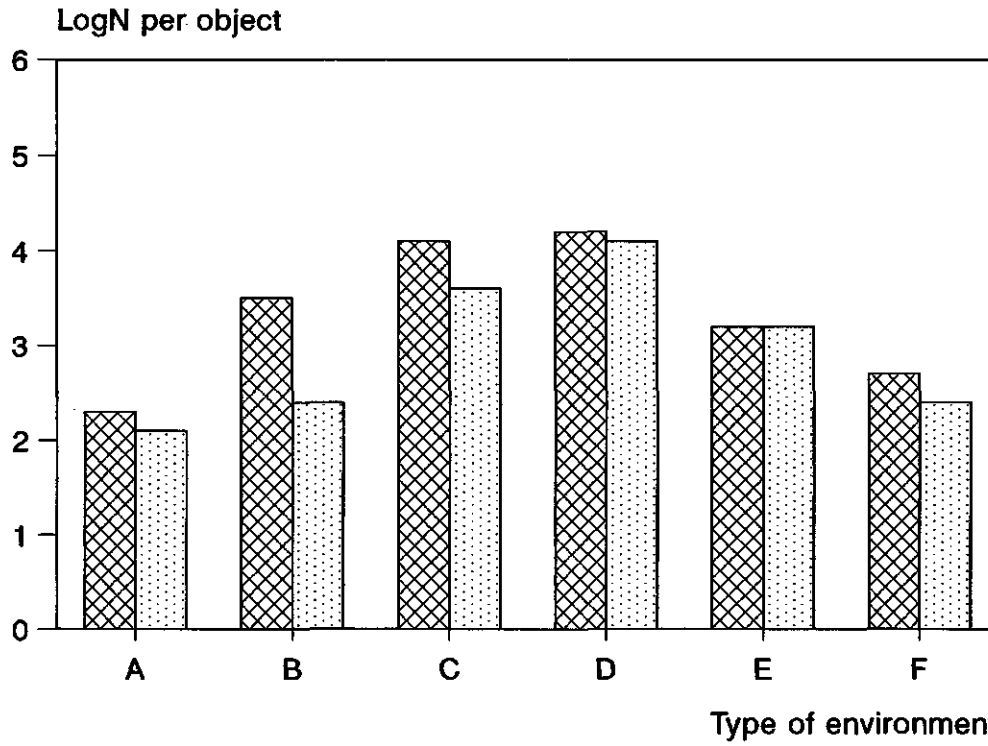

Fig. 1 Mean values of *Listeria* spp. in domestic environments (LogN/object). Surface samples (20 cm^2) of the vegetable compartment of refrigerators (A), the kitchen sink (B, around the drain), the bathroom (F, around the drain of showers) and rinse samples of washing-up brushes (C), dish-cloths (D) and tooth-brushes (E) were investigated (*L. monocytogenes* chequered bars, *L. innocua* stippled bars)

were present in two samples and in 4 houses (4%) *Listeria* occurred in three of the samples analysed (data not shown). Neither the number of residents in a house (1-9), nor the time of sampling (morning or afternoon) influenced the contamination rate of the sampling sites (data not shown).

DISCUSSION

Since *Listeria* spp. are commonly found in the environment (Weis and Seeliger 1975), the presence of these organisms in the domestic environment is not surprising. In previous work 20% of the kitchens in 35 households were found to be contaminated

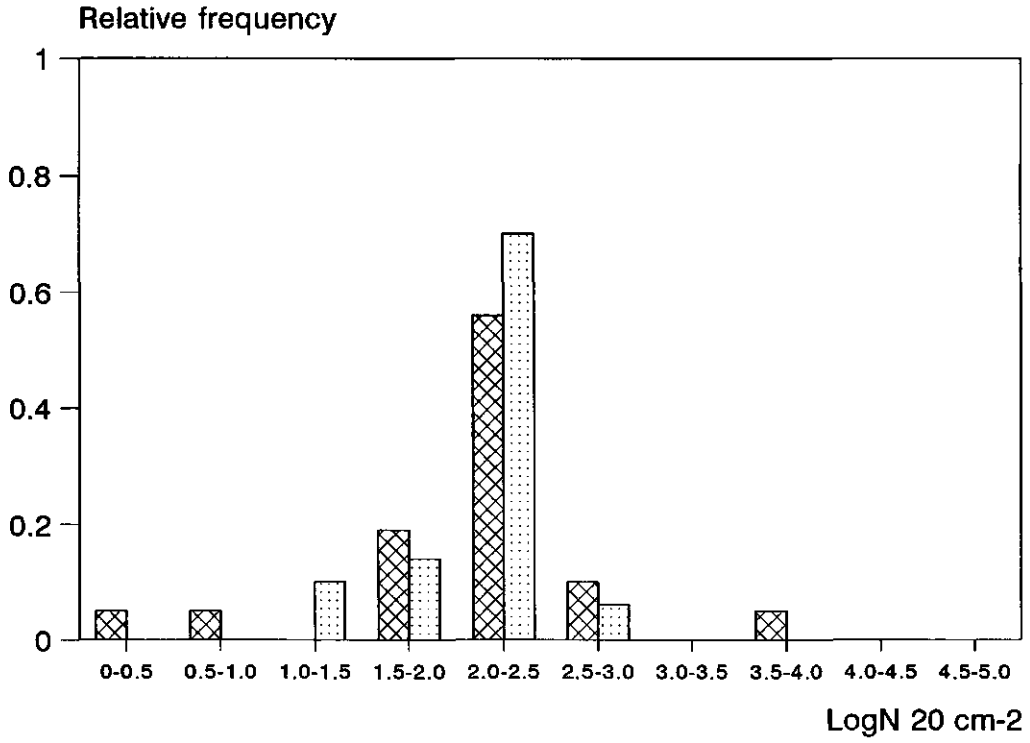

Fig. 2a Relative frequency distribution of *Listeria* spp. in bathrooms, 20 cm^2 surface samples around the drain of showers (*L. monocytogenes* chequered bars, *L. innocua* stippled bars)

with listerias (Cox et al. 1989). Almost any moist environment will probably support survival and growth of these and other microorganisms, if substrates are available. However, the extent to which products or people become contaminated will, amongst others, depend on the numbers of microorganisms present. Unfortunately, most studies detecting pathogens employ enrichment protocols. Therefore little quantitative information is available in the literature.

From the experiments with artificially contaminated dish-cloths, tooth-brushes and washing-up brushes, it was shown that with the methods we used 75-120% of the initial flora could be recovered. The rather high standard deviations (up to 26%) may be due

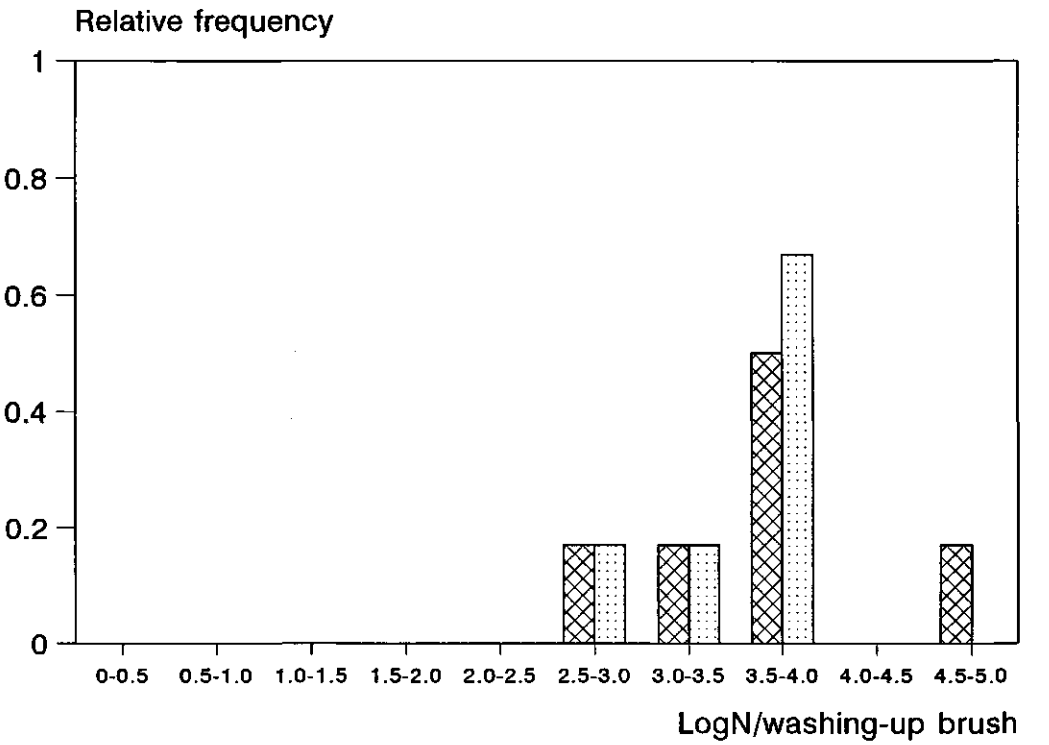

Fig. 2b Relative frequency distribution of *Listeria* spp. on washing-up brushes (*L. monocytogenes* chequered bars, *L. innocua* stippled bars)

to the isolation technique used. Vigorously shaking of the stomacher bags by hand is in all probability less effective than the use of mechanical equipment. On all objects the test strain was detectable after 24 h at room temperature. On new objects numbers of *L. monocytogenes* were slightly lower compared to the numbers on old objects. This may be due to a lack of food, or to the presence of inhibitory substances (Daley et al. 1995). The slight growth indicates that, in case of bad hygienic practices, outgrowth to high numbers may occur.

Table 2 shows that *Listeria* spp. were isolated from 101 (47.4%) of the 213 households investigated. In 45 (21.1%) of the houses investigated *L. monocytogenes* was

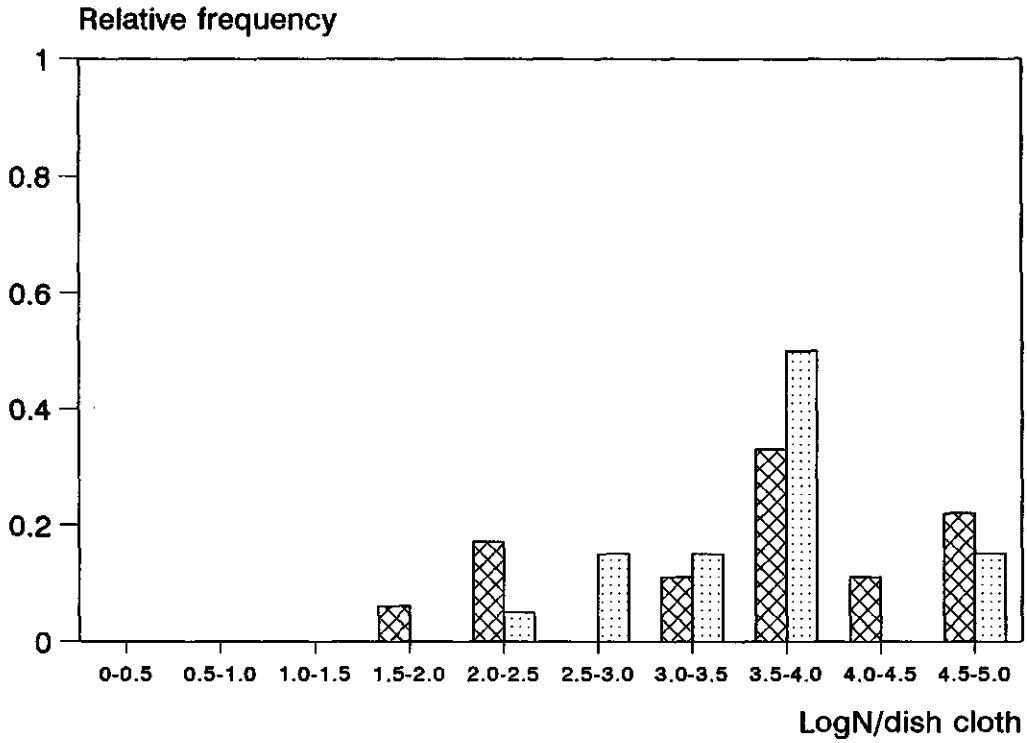

Fig. 2c Relative frequency distribution of *Listeria* spp. on dish-cloths (*L. monocytogenes* chequered bars, *L. innocua* stippled bars)

present. Although this is an increase in comparison with the data of Cox et al (1989), it remains uncertain if this is due to the increase in sampling sites numbers of houses investigated, or to differences in the isolation protocols for *Listeria*. Since in this study isolation of *Listeria* spp. was performed using a direct (selective) plating procedure, other listerias or competitive microorganisms could not mask the presence of *L. monocytogenes* (Curiale and Lewus 1994). Different *Listeria* spp. were isolated from one household, but in positive samples only one *Listeria* sp. was detected.

Confirmation and subsequent identification of more than three typical colonies on the plates might have led to the detection of more than one *Listeria* sp. in the positive samples. However, it is likely that growth of *Listeria* at the sampling sites is comparable to the growth in enrichment broths. Fast growing listeria's (adapted to the ecological niche) will mask the presence of slower growing species.

Highest contamination levels were found in wet environments such as dish-cloths (37%) and in surface samples from the bathroom (27.2%). On the contrary, samples taken around the drain in the kitchen sink had a significant lower contamination level (5.7%). This may be partly due to the type of material, stainless steel for the kitchen sink and tiles in the bathroom. In addition, kitchen sinks are more often in contact with warm or hot water containing detergents (washing-up), which may drastically reduce numbers of listerias present.

Washing-up brushes and tooth brushes are wetted only a few times a day. During shorter or longer periods, depending on the type and the degree of corrosion (cracks) of the material, the storage conditions, and the presence of organic matter, growth may occur. After the main part of the water has been evaporated, growth will stop and after a period of survival die off may set in. This probably accounts for the lower contamination level of washing-up brushes (12.5%) and tooth brushes (8.5%), compared with dish-cloths (37%).

In only 3.9% of the 204 refrigerators investigated, listerias were detected in the vegetable compartment. In previous work similar results, one (3%) positive of 35 refrigerators sampled, were obtained by Cox et al. (1989), whereas this pathogen was not isolated by Jackson et al. (1993). A possible explanation for these low contamination rates of vegetable compartments in refrigerators may be that these parts of the refrigerator are likely to be cleaned frequently, since they often become visibly soiled. Moreover, the growth of the organisms may be limited by the low relative humidity as a result of the removal of water from the air by condensation and freezing. It has been

reported that on inert and dry surfaces at room temperature, the number of viable *L. monocytogenes* cells decreased. Although the organisms could still be recovered after 4-6 weeks (Welshimer 1960, Dickgießer 1980).

The present study indicates that *L. monocytogenes* and *L. innocua* are the predominant *Listeria* spp. in domestic environments (Table 2; Fig. 2a-2c). All listerias, including *L. monocytogenes*, were commonly found in high numbers at wet places (Table 2). The frequent occurrence in high numbers suggests that listerias may be actively growing and that hygienic practices in domestic kitchens could be improved. Humans are exposed to this pathogen on a regular basis, because of its ubiquity in food products and the environment (Farber and Peterkin 1991). However, until now there was no evidence that domestic sources of *L. monocytogenes* play an important role in the contamination of people and their foods.

In previous studies on microbial contamination in the domestic environment (Finch et al. 1978, Scott et al. 1982), dish-cloths were noted as being sources of contamination with *Enterobacteriaceae* in approximately 45-50% of homes examined. Investigations for listerias were not included in these studies. From our data it becomes clear that *L. monocytogenes* may be present in high numbers in the domestic environment. It must be borne in mind that even low numbers of *L. monocytogenes*, may proliferate and become hazardous if transferred to food products with a long (refrigerated) shelf-life such as cooked meat products and cheeses with a surface flora. Other workers have recognized the potential for spread of contamination by cleaning utensils and the persistence of microorganisms in the environment (Davis et al. 1968, Westwood et al. 1971, De Wit et al. 1979).

Although outbreaks of listeriosis have been associated with foods, in future epidemiological studies of the domestic environment should also be taken into account. This may reveal if these sources contribute to outbreaks of listeriosis. From the results of this study it seems that careful personal and domestic hygiene could be an important factor in the prevention of human listeriosis.

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GENERAL DISCUSSION

RR Beumer

INTRODUCTION

Listeria monocytogenes is a widely distributed bacterial pathogen, that has been isolated from a wide variety of foods, including meat, poultry, dairy, marine and vegetable products. The ubiquity of the organism and its ability to grow at refrigeration temperatures present distinct problems to the food industry. In this thesis factors influencing the isolation, confirmation and identification of *L. monocytogenes* were investigated and the behaviour in food and environment was studied.

DETECTION OF *LISTERIA MONOCYTOGENES*

There are three basic protocols for the detection of pathogens: 1) direct plating on selective media, 2) direct selective enrichment and 3) pre-enrichment. Depending on the number of cells expected in a sample and/or the standards described (by law or otherwise), one or more of these procedures may be used for their detection. The success of all three basic protocols depends on 1) the number and the state of the microorganisms in the sample, 2) the selectivity of the media (a balance between inhibition of competitors and inhibition of the target organism), 3) conditions of incubation (time, temperature, presence of oxygen) and 4) the electivity of the isolation medium (the ease of distinction between the target organism and competitive microflora).

Of the six accepted species of *Listeria*, only *L. monocytogenes* is a human pathogen. Generally, microorganisms of the same genus are not equally affected by inhibitors used in selective enrichment media. Therefore, in procedures where all species of a genus are considered to be pathogen, e.g. *Salmonella*, only epidemiologically false information may be obtained. However, in case of detecting *L. monocytogenes*, other (faster growing) listerias can mask the presence of this pathogen (MacDonald and Sutherland 1994) (Chapter 1). The use of lithium chloride in the enrichment procedure did not overcome this effect (Chapter 2). Further studies revealed that the use of acriflavine in enrichment media had both direct and indirect effects on the isolation of *L. monocytogenes*. Increasing acriflavine concentrations affected both lag time and generation time of *L. monocytogenes*, while hardly any effect was observed on *L. innocua*. Acriflavine binds to proteins in the samples, and with decreasing pH more acriflavine was bound. Growth promoting effects for *L. monocytogenes* are limited, because growth of this pathogen is restricted at low pH (Chapter 3). It seems that the competitive microflora benefits more, since it has been reported that the predominant microflora in enrichment media for *Listeria* consists of lactobacilli and enterococci (Duffy et al. 1994).

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All commercially available isolation media for *Listeria* suffer from the same disadvantage: *L. monocytogenes* cannot be differentiated from non-pathogenic listerias. Even the selection of five colonies from such media does not guarantee the detection of this pathogen, even though a few *L. monocytogenes* colonies are present on the plate. Therefore, enhanced hemolysis agar (EHA), a medium on which *L. monocytogenes* can be distinguished from other *Listeria* spp. on the basis of hemolysis, previously described by Cox et al. (1991a 1991b) was improved (Chapter 4). The use of EHA as direct plating- or isolation medium is to prefer to the use of media applying an upper layer with blood agar, because this doubles the incubation period.

The traditional methods for the detection of *Listeria* are both time consuming and labour intensive. Therefore, food producers and distributors as well as public health authorities have a great interest in rapid methods. It was shown that there are some *Listeria* and *L. monocytogenes* test kits on the market, which may be superior to the standard methods (Chapter 5). The results were strongly dependent on the concentration of *Listeria* cells in the sample, the detection limits of the tests and the enrichment protocol used. Most rapid tests detected all samples with high numbers of listerias (>10 cfu g^{-1}). Because the outcome of collaborative and comparative studies is often used for promotion purposes, results obtained with samples with high numbers of *Listeria* should be excluded. Moreover, the use of identical enrichment protocols is recommended. In those cases where this is not feasible, cross-checking of the enrichment media is necessary (Noah et al. 1991). There are rapid tests for the detection of *Listeria* spp. as well as for the detection of *L. monocytogenes*. It is difficult, if not impossible to prefer one to another. Only methods detecting the human pathogen *L. monocytogenes* may be preferably. However, knowing the masking of *L. monocytogenes* in enrichment media by other listerias, it would be wise to opt for the detection of the genus *Listeria*.

For confirmation of typical colonies on isolation media, microscopy (Gram-stain) and traditional biochemical tests are used (Lovett 1988). These tests can also be replaced by rapid methods, such as latex tests and DNA-probes. For identification of *Listeria* spp., systems based on biochemical tests are available. All confirmation and identification tests used in this study performed well (Chapter 6). For epidemiological studies further typing of the strains is necessary. Both serotyping (Seeliger and Höhne 1979) and phage-typing (Rocourt et al. 1985) are commonly used for this purpose. Unfortunately, serotyping provides insufficient information (only three serotypes are responsible for the majority of listeriosis outbreaks) and, although phage typing allows further strain

subdivision, not all isolates are typable with the existing set of phages. Moreover, only a small number of laboratories have the possibility to use this method. Multilocus enzyme electrophoresis (Harvey and Gilmour 1994) and DNA-based typing methods such as restriction fragment length polymorphism (RFLP) (Ridley 1995), randomly amplified polymorphic DNA (RAPD) (Farber and Addison 1994) and ribotyping (Wiedmann et al. 1996) are valuable alternatives for traditional typing protocols (Nørrung and Gerner-Smidt 1993, Boerlin et al. 1995).

BEHAVIOUR OF *L. MONOCYTOGENES*

Several studies have reported conflicting results concerning the survival and growth of *L. monocytogenes* on meat. Due to the use of different strains (Barbosa et al. 1995), inoculated on various types of meat, and storage temperatures varying from 0-30°C in different atmospheres, results cannot be compared directly (Buchanan and Klawitter 1991, Kaya and Schmidt 1991). In this study it was shown that growth of *L. monocytogenes* on meat was strain dependent and mainly determined by initial pH and storage temperature (Chapter 8). Abusive storage temperatures may result in the growth of this pathogen. For that reason, and because survival at low pH for a long period is likely (Farber and Peterkin 1991), raw meat that has been stored for a long time, should not be used for the production of ready-to-eat products such as steak tartare.

In cooked meat products *L. monocytogenes* should be absent. As raw meats generally contain low numbers of *Listeria* (less than 100 cfu g^{-1}), the heating of these products for 2 min at 70°C, or to core temperatures of 71°C, is likely to inactivate any *L. monocytogenes* present (Mackey et al. 1990). Still the incidence of *L. monocytogenes* on prepacked sliced cooked meat products has frequently been reported. From previous studies it is clear, that presence of this pathogen was most likely due to post process contamination rather than survival of the heating process (Wang and Muriana 1994). As these products are supposed to have a refrigerated shelflife of several weeks, it is important to know the growth potential of this pathogen on cooked meat products. In the Netherlands luncheon meat, ham and cooked chicken breast were the most frequently contaminated cooked meat products (De Boer 1990). In this study (Chapter 9) it was shown that during the shelf life (4-6 weeks) of artificially contaminated sliced cooked meat products, the growth of *L. monocytogenes* in vacuum packaged products was similar to the growth in modified atmosphere (30% CO_2/70% N_2) packed products.

Despite improved transport of food products (fast, controlled conditions), there is a tendency towards extending storage life of raw or minimally processed foods through cooling and modified atmosphere packaging (MAP). The predominant microorganisms on the cooked meat products in this study were lactic acid bacteria. Even if lactic acid bacteria are present in concentrations one hundred times those of *L. monocytogenes*, the growth of this pathogen was only slightly inhibited. At the end of the shelflife, levels were still 10^7 cfu g^{-1}. To prevent outgrowth of *L. monocytogenes* to such high levels, it is necessary to prevent recontamination during slicing and packaging, and to shorten the rather long shelflife of these products. Addition of nisin in a concentration of 25 mg per kg to luncheon meat, led to an initial decrease in numbers of *L. monocytogenes*. After storage for one week at 7°C, the levels were about two log units lower compared to the controls, but at the end of the storage time (after 6 weeks) counts in both products exceeded 10^7 cfu g^{-1}. This phenomenon is due to the rapidly decreasing activity of nisin in food products (Chung et al. 1989, Fang and Lin 1994).

Since spoilage may not be evident in MAP foods, consumers could judge such products as safe even in the presence of high numbers of pathogens. Processors should be aware of this and take measures to prevent recontamination of the products. Studies in factory ecology can be a valuable tool for this. As such steps will never fully guarantee the absence of a single pathogen, the shelflife of products supporting the growth of *L. monocytogenes* and/or other pathogens should be restricted to the period necessary for an initial contamination of *L. monocytogenes* to reach numbers of 10^2 cfu g^{-1} at the last day of consumption. In respect of this, challenge studies will be necessary with strains preferably isolated from similar naturally contaminated products, or from food processing environments (Notermans and In 't Veld 1994).

As the recontamination of food products with *L. monocytogenes* is usually limited, only few (micro) colonies will be formed during the storage. Therefore, it is recommended to examine large portions (e.g. whole packages) to increase the chance to detect *L. monocytogenes*.

L. monocytogenes can grow in the presence of a wide range of salt concentrations in complex media and food products. To understand the ability of a microorganism to grow and survive in widely diverse environments, it is necessary to determine the nutritional requirements of that organism in a chemically defined minimal medium (MM). The MM is also important to study how these nutritional requirements are affected under various stress conditions. In this study a MM that was based on a

medium described by Premaratne et al. (1991), containing five essential amino acids, was used to determine the influence of incubation temperature, salt concentration and various osmoprotectants on the growth of *L. monocytogenes* (Chapter 10). In the absence and presence of NaCl (3 %) final cell numbers reached in minimal medium were 10^9 and 10^7 cfu ml^{-1}, respectively. Growth in the latter condition could not be detected by spectrophotometry measuring absorbance at 660 nm. Apparently this technique is not suitable for these experiments since the detection level is $>10^7$ cfu ml^{-1}.

Exogenously added proline, betaine and carnitine significantly stimulated growth under osmotic stress conditions in minimal medium both at 37 and 10°C. Betaine and carnitine are present in foods derived from plant and animal origin, respectively. These compounds can therefore contribute significantly to growth of *L. monocytogenes* in various foods at high osmolarities.

Although various food products have been implicated in outbreaks of listeriosis, the source and route of infection are usually unknown. The investigation of a case of listeriosis is often hampered by both the limitations of traditional typing techniques and by the occurrence of strains unrelated to the outbreak strain in implicated food products and in the environment (Kerr et al. 1995). This can be contributed to the isolation methods used. However, it cannot be ruled out that unknown sources of *L. monocytogenes* may also be responsible for illness. Although presence of *Listeria* spp. in household environments has been demonstrated (Cox et al. 1989), these environments have not frequently been included in epidemiological studies. In this study (Chapter 11) the occurrence of *Listeria* spp. was investigated in 213 households in The Netherlands. Samples were taken from six domestic environments such as dishcloths, washing-up brushes, tooth brushes, the bathroom (shower), the kitchen sink and the vegetable compartment of the refrigerator. Using a direct isolation method *Listeria* spp. were detected in 101 (47.4%) of the 213 houses investigated. In 45 (21.1%) *L. monocytogenes* was present. *Listeria* occurred at all sampling sites; dishcloths (37%) and surface samples around the drain in the bathroom (27.2%) were most frequently contaminated. Highest numbers (10^4-10^5 cfu/object) were found in dishcloths and washing-up brushes. From these data it is clear that *L. monocytogenes* may be present in high numbers in the domestic environment. Until now it is not clear if these numbers contribute directly or indirectly (via contamination of food by these objects) to listeriosis. Future epidemiological studies should also take the domestic environment into account. The use of appropriate typing methods may elucidate if these sources contri-

bute to outbreaks of listeriosis. From the results of this study it seems that careful personal and domestic hygiene could be an important factor in the prevention of human listeriosis and other foodborne infections.

CONCLUSION

Fortunately, the numbers of listerias in most food products are low ($< 10^2$ cfu g^{-1}). However, in ready-to-eat products with a long refrigerated shelf life numbers may increase to 10^6-10^8 cfu g^{-1}. As the pathogenicity of *L. monocytogenes* strains varies considerably (Hof and Rocourt 1992) and the susceptibility for this pathogen may vary from individual to individual, it remains obscure which dose is necessary to cause illness and which microbiological standards will guarantee safe food.

Food safety can be defined as: 'All conditions and measures necessary in the whole food chain (from production to consumption), to ensure that food is a negligible risk to health'. It is currently considered that ready-to-eat-foods should be free from salmonellas, campylobacters, *Escherichia coli* O157 and other verotoxin producing strains of *E. coli* (VTEC). For the remaining pathogens (*Bacillus cereus*, *Clostridium perfringens*, *L. monocytogenes*) generally more liberal standards or guidelines are given.

The largest US listeriosis outbreak occurred in 1985 in Southern California (Linnan et al. 1988) and led to the formulation of the 'zero tolerance' for *L. monocytogenes* in ready-to-eat foods, adopted first by the Food and Drug Administration (FDA) and then by the US Department of Agriculture (USDA) (Klima and Montville 1995). More realistic criteria, including a sample plan, have been formulated by the International Commission on Microbiological Specifications for Food (Van Schothorst 1994). However, no sampling plan can ensure the absence of a pathogen in food (ICMSF 1986). The government, industry and consumer are the most important parties in ensuring food safety. The government should establish standards and develop codes of practice and ensure that these will be observed. Food producers and food preparators should produce safe food, which may be achieved by adequate hygiene standards, good manufacturing practices (GMP) and implementation of hazard analysis and critical control points (HACCP). Consumers, in particular those who buy food and prepare meals, should have a basic knowledge of safe food preparation.

Due to the great variety in protocols and media for the detection of *Listeria* and/or *L. monocytogenes* it is difficult to make the right choice. Fortunately, the proposed ISO method (Anonymous 1995) employs a medium that keeps its pH at neutral values during

enrichment. However, one of the major drawbacks is the rather high (37°C) incubation temperature of the secondary enrichment broth. Many immunological tests are based on flagellar antibodies, and since *Listeria* spp. loose their motility at 37°C, it would be better to prescribe an incubation temperature of 30°C both for primary and secondary enrichment.

Presence of *L. monocytogenes* in heat-processed food products is due to recontamination rather than to survival of heat treatments. Therefore, not too much attention should be paid to the recovery of injured cells. Many studies indicating the presence of damaged cells, leading to lower recoveries on selective isolation media, were performed with unrealistically high initial numbers of *Listeria*. To control presence of *L. monocytogenes* in food knowledge of factory ecology is as important as knowledge about microorganisms obtained in challenge experiments and both are indispensable in HACCP procedures. Many researchers are convinced that quantitative risk analysis also contributes to a lower incidence of listeriosis and other foodborne infections (Notermans and Jouve 1995, Todd and Harwig 1996). Certainly, hazard identification, followed by risk characterization (to estimate the severity of the hazard) are essential steps in risk management. However, obtaining reliable data for exposure assessment and dose response assessment is difficult, if not impossible. After foodborne outbreaks the numbers of pathogens in the incriminated products may have changed, due to outgrowth or die-off. Moreover, the detection methods used may hamper, due to high numbers of competitors or otherwise.

It has been suggested that the decreased incidence of listeriosis in the UK (McLauchlin 1992), Spain (Nolla-Salas et al. 1994) and in the USA (Tappero et al. 1995) can be contributed to the effectiveness of the preventive measures (zero tolerance, intensified clean-up programs and dietary recommendations). Therefore, international microbiological criteria (preferably standards, since guidelines are non-committal) should be established. As zero-tolerance for ready-to-eat foods is unrealistic, international agreement should be based on $<10^2$ *L. monocytogenes* cells per gram on the sell-by date. Persons at risk (immuno-compromized, pregnant) should be strongly advised not to eat food products likely to contain *L. monocytogenes*. Meanwhile, more attention should be given to challenge studies, factory ecology and sound cleaning and disinfection. From studies to evaluate dairy and food plant sanitizers it was concluded that many commonly used sanitizers are effective at the recommended concentration. However, particularly in

the presence of organic material not all disinfectants perform equally well against *Listeria* spp. (Best et al. 1990).

In conclusion, the problems caused by *L. monocytogenes* seem controllable, in contrast with these caused by *Salmonella* and *Campylobacter*.

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SUMMARY

Listeria monocytogenes is the causative agent of listeriosis, a disease with a high mortality to susceptible individuals (immuno-comprimized, pregnant, extremes of age). In the past 10-15 years listeriosis has emerged as a foodborne illness in a series of large outbreaks from contaminated milk, coleslaw, soft cheese and pâté. The organism is ubiquitous in the environment, and has been detected in a variety of raw and ready-to-eat food products.

Isolation, identification and further characterization of *Listeria* spp. from food and food production environments are common and essential aspects of food hygiene. Unfortunately, in the enrichment procedures for *L. monocytogenes*, other (faster growing) listerias can mask the presence of this pathogen. A first attempt to overcome this problem, the use of an enrichment medium based on lithium chloride and nalidixic acid, is described in Chapter 2. *L. monocytogenes* could grow at concentrations up to 2% LiCl, but above this level, inhibition was observed. The competitive microflora capable of growth in the presence of lithium chloride is normally inhibited by nalidixic acid, except for staphylococci. Further studies with this medium were abolished, because this medium suffered from the same disadvantage as the other media: masking of *L. monocytogenes* by other *Listeria* spp.

Instead of this, the effect of acriflavine on the growth of *Listeria* spp. was studied. In Chapter 3 it is concluded that the use of acriflavine in enrichment media for *Listeria* spp. has both direct and indirect effects on the isolation of *Listeria monocytogenes*. Increasing acriflavine concentrations affect both lag time and generation time of *L. monocytogenes*, whereas hardly any effect was observed on *L. innocua*. As acriflavine binds to protein in the samples, the acriflavine activity decreases. This lower activity may result in a better growth of *L. monocytogenes*. At low pH-values ($pH < 5.8$) more acriflavine is bound, but growth promoting effects are limited, because growth of this pathogen is restricted at low pH. On account of this, one may expect that enrichment protocols employing low acriflavine concentrations with an adequate buffer, favour the isolation of *L. monocytogenes*. Because comparative studies have paid no attention to the ratio *L. monocytogenes* to other *Listeria* spp., virtually nothing is known about the inferior detection of *L. monocytogenes*. For that reason, it is worthwhile introducing an isolation medium that facilitates identification of *L. monocytogenes* in the presence of high numbers of other listerias in enrichment protocols.

Enhanced hemolysis agar (EHA) is a good alternative for other isolation media, because a few *L. monocytogenes* colonies can be detected between a majority of colonies of other listerias on basis of hemolysis. In Chapter 4 studies to optimize the haemolysis reaction in this medium are described.

In Chapter 5 it has been shown that there are some *Listeria* and *Listeria monocytogenes* rapid test kits on the market which are superior to the standard USDA method. Considerable differences exist among various test kits, which may be contributed to the enrichment protocols, the detection limits of the tests and the concentration of *Listeria* cells in the samples investigated. Most rapid tests detected all samples with high numbers (>10 cfu g^{-1}) of listerias. As these samples can be considered as 'positive controls', these results should not be emphasized in the evaluation of the method. In collaborative and comparative studies, (rapid) methods are sometimes compared to standard protocols using completely different enrichment protocols. The use of identical protocols is recommended. In those cases where this is not feasible, cross-checking of the enrichment media is necessary.

Typical colonies on isolation media for *Listeria* should be confirmed by Gram-stain and traditional biochemical methods. In Chapter 6 it was concluded that commercially available rapid tests for the confirmation and identification of *Listeria* or *L. monocytogenes* can replace the traditional methods.

In the second part of this thesis, the behaviour of *L. monocytogenes* in food and environment was studied.

Conflicting results about the survival and growth of *L. monocytogenes* on meat are reported in literature. Due to the use of different strains, inoculated on various types of meat, and stored at temperatures varying from 0-30°C in different atmospheres, results cannot be compared directly. In Chapter 8 it was shown that growth of *L. monocytogenes* on raw meat was strain dependent and mainly determined by initial pH and storage temperature. However, abusive storage temperatures may result in the growth of this pathogen within the prescribed shelflife.

The incidence of *L. monocytogenes* on cooked meat products (generally due to recontamination) has frequently been reported. In Chapter 9 it is reported that during the shelflife (4-6 weeks) of artificially contaminated cooked meat products the growth of *L. monocytogenes* in vacuum was similar to the growth in modified atmosphere (30% CO_2/70% N_2) packaged products. The presence of competitors (lactic acid bacteria), even in concentrations one hundred times those of *L. monocytogenes*, only slightly

inhibited growth of this pathogen. At the end of the shelflife, levels were still 10^7 cfu g^1. Because spoilage may not be evident in modified atmosphere packaged foods, consumers would judge such products as safe even in the presence of high numbers of *L. monocytogenes*. To prevent outgrowth of *L. monocytogenes* to such high levels it is necessary to prevent recontamination during slicing and packaging, and to shorten the rather long shelflife of these products.

The influence of incubation temperature, salt concentration and various osmoprotectants on the growth of *L. monocytogenes* in a minimal medium are described in Chapter 10. In the absence and presence of NaCl (3 %) final cell numbers reached in minimal medium were 10^9 and 10^7 cfu ml^{-1}, respectively. Growth in the latter condition could not be detected by spectrophotometry measuring absorbance at 660 nm. Apparently this technique is not suitable for these experiments since the detection level is $>10^7$ cfu ml^{-1}. Exogenously added proline, betaine and carnitine significantly stimulated growth under osmotic stress conditions in minimal medium both at 37 and at 10°C. Betaine and carnitine are present in foods derived from plant and animal origin, respectively. These compounds can therefore contribute significantly to growth of *L. monocytogenes* in various foods at high osmolarities.

Although various food products have been implicated in outbreaks of listeriosis, the source and route of infection are usually unknown. The investigation of a case of listeriosis, is often hampered by both the limitations of traditional typing techniques and by the occurrence of strains unrelated to the outbreak strain in implicated food products. As unknown sources of *L. monocytogenes* may also be responsible for illness, domestic environments were investigated for the presence of *Listeria* spp. In Chapter 11 it is shown that with a direct isolation method *Listeria* spp. were detected in 101 (47%) of the 213 houses investigated. In 45 (21.1%) of the houses investigated *L. monocytogenes* was present. *Listeria* occurred at all sampling sites: dishcloths (37%) and surface samples round the drain in the bathroom (27%) were most frequently contaminated. Highest numbers (ca 10^4 cfu/object) were found in dishcloths and washing-up brushes. In total, 132 *Listeria* spp. were isolated from 871 samples. *L. innocua* (53%) and *L. monocytogenes* (41%) were the predominant species in the positive samples. From these results it seems that careful personal and domestic hygiene could be an important factor in the prevention of human listeriosis.

This thesis contributes to a better knowledge of the detection of *L. monocytogenes* and the incidence and behaviour of this pathogen in foods and in the environment.

SUMMARY

SAMENVATTING

Listeria monocytogenes is de veroorzaker van listeriose, een ziekte die in veel gevallen tot sterfte leidt, met name bij zwangeren en personen met een verzwakte afweer (aids- en kankerpatiënten, bejaarden en pas geborenen). Vanaf de 80-er jaren is er een aantal grote uitbraken van listeriose geregistreerd, veroorzaakt door o.a. besmette melk, koolsla, zachte kaas en pâté. *L. monocytogenes* komt algemeen voor en wordt in vrijwel alle rauwe en kant en klare produkten aangetroffen.

Aanwezigheid van *L. monocytogenes* in levensmiddelen wordt meestal aangetoond door gebruik te maken van ophopingsprocedures, gevolgd door nadere identificatie m.b.v. biochemische, immunologische of DNA-methoden. Een belangrijk nadeel bij het ophopen van *L. monocytogenes* is dat andere, niet-pathogene listeria's sneller groeien: hierdoor wordt de pathogeen niet aangetroffen op het isolatiemedium. In Hoofdstuk 2 is een nieuw ophopingsmedium beschreven, met lithiumchloride en nalidixinezuur als remstoffen voor de begeleidende flora. Concentraties lithiumchloride tot 2% hebben geen effect op de groei van *L. monocytogenes*, maar hogere concentraties remmen de groei van dit micro-organisme. De begeleidingsflora die zich ontwikkelt in aanwezigheid van lithiumchloride werd, staphylococcen uitgezonderd, geremd door nalidixinezuur. Omdat ook in dit ophopingsmedium *L. monocytogenes* werd overgroeid door andere listeria's, werd het onderzoek met dit medium niet voortgezet.

Om vast te stellen waarom *L. monocytogenes* meestal langzamer groeide dan de andere listeria's in de gebruikelijke ophopingsmedia, werd het effect van acriflavine op de groei van *Listeria* onderzocht (Hoofdstuk 3). Het bleek dat hogere concentraties acriflavine zowel de lagfase als de deeltijd van *L. monocytogenes* verlengden. Op andere listeria's was dit effect veel geringer. Bovendien bleek dat acriflavine gebonden wordt aan eiwitten in de onderzochte monsters. Bij lage pH-waarden ($pH < 5.8$), in slecht gebufferde ophopingsmedia, wordt meer acriflavine gebonden. Hoewel het remmende effect van acriflavine op *L. monocytogenes* hierdoor wordt verminderd, leidt dit niet tot betere groei, omdat groei van dit micro-organisme door de verlaagde pH beperkt is. Uit het onderzoek blijkt dat goed gebufferde ophopingsmedia met een lage concentratie aan acriflavine het meest geschikt zijn voor het isoleren van *L. monocytogenes*. Omdat de kiem ook dan nog overgroeid kan worden door andere listeria's, verdient het aanbeveling een isolatiemedium te gebruiken, dat het mogelijk maakt *L. monocytogenes* te onderscheiden van niet-pathogene listeria's.

Enhanced hemolysis agar (EHA) is een medium waarop zelfs enkele kolonies van *L. monocytogenes* (door hun hemolyse-activiteit) waargenomen kunnen worden tussen tientallen kolonies van andere listeria's en is daarom een goed alternatief voor bestaande isolatiemedia. In Hoofdstuk 4 is beschreven hoe de hemolyse-reactie in dat medium verbeterd kon worden.

Er zijn diverse 'snelle' testen voor het aantonen van *Listeria* of *L. monocytogenes* verkrijgbaar. In Hoofdstuk 5 worden deze testen vergeleken met de traditionele USDA methode. Het blijkt dat de 'snelle' testen vooral goed presteren als hoge aantallen (>10 kve g^{-1}) listeria's in de monsters aanwezig zijn. Op grond hiervan wordt aangeraden bij het vergelijken van testen, of bij ringonderzoeken, de resultaten verkregen met deze monsters niet te benadrukken, omdat deze monsters als 'positieve controle' te beschouwen zijn. Bovendien wordt voorgesteld om bij het vergelijken van 'snelle' testen uit te gaan van identieke ophopingsprotocollen. Als dat niet mogelijk is, dan moeten alle testen gebruikt worden in combinatie met elk protocol, om mediumeffecten uit te sluiten.

Verdachte kolonies op isolatiemedia moeten, na reinstrijken, bevestigd worden met een Gramkleuring en traditionele biochemische reacties. Uit Hoofdstuk 6 blijkt dat hiervoor ook commerciëel verkrijgbare snelle testen gebruikt kunnen worden.

Het tweede deel van dit proefschrift gaat over het gedrag van *L. monocytogenes* in voedsel en in de omgeving.

Er zijn tegenstrijdige resultaten gepubliceerd over de groei van *L. monocytogenes* op vlees. Dit komt vooral door het gebruik van steeds andere stammen, verschillende soorten vlees en bewaartemperaturen (bij diverse gassamenstellingen) die variëerden van 0-30°C. In Hoofdstuk 8 is aangetoond dat de groei van *L. monocytogenes* op rauw vlees afhangt van de gebruikte stam en verder voornamelijk wordt bepaald door de pH van het vlees en de bewaartemperatuur.

Door herbesmetting kan *L. monocytogenes* voorkomen op gekookte vleeswaren. Met behulp van kunstmatig besmette vleeswaren is aangetoond dat de groei van deze pathogeen in gasverpakte produkten (30% CO_2/70% N_2) vergelijkbaar is met de groei in vacuumverpakte produkten (Hoofdstuk 9). Begeleidende micro-organismen (melkzuurbacteriën), zelfs als de aantallen honderd maal hoger waren dan die van *L. monocytogenes*, hadden slechts een gering effect op de groei van deze ziekteverwekker. Op het einde van de houdbaarheid (na 4-6 weken) waren de aantallen toch nog 10^7 kve g^{-1}. Omdat de bederfflora van gasverpakte vleeswaren voornamelijk bestaat uit melkzuur-

bacteriën, wordt bederf niet makkelijk waargenomen. Om hoge aantallen *L. monocytogenes* te voorkomen moet herbesmetting zoveel mogelijk worden voorkomen en moet de houdbaarheid van deze produkten verkort worden.
In Hoofdstuk 10 is het effect van bebroedingstemperatuur, zoutconcentratie en stoffen die groei mogelijk maken bij hoge zoutconcentraties (osmoprotectantia) op *L. monocytogenes* beschreven in een minimaal medium. Toevoegen van 3% NaCl aan het minimaal medium verminderde het uiteindelijke aantal cellen van 10^9 tot 10^7 kve ml^{-1}. Groei in aanwezigheid van zout kon niet met de spectrofotometer worden bepaald, omdat hiervoor het aantal benodigde cellen hoger dan 10^7 ml^{-1} moet zijn. Toevoegen van proline, betaine en carnitine resulteerde in een significant betere groei, zowel bij 10 als bij 30°C. Betaine (plantaardige produkten) en carnitine (dierlijke produkten) kunnen in belangrijke mate bijdragen aan de groei van *L. monocytogenes* in produkten met hoge zoutconcentraties.

Hoewel in het verleden verschillende levensmiddelen betrokken waren bij uitbraken van listeriose, zijn de bronnen van besmetting en de besmettingswegen vaak onbekend gebleven. Dit lag vaak aan de gebruikte isolatie- en typeringsmethoden en aan het voorkomen van stammen die niet overeenkomen met de stam die de ziekte veroorzaakte. Het is dus mogelijk dat onbekende bronnen van *L. monocytogenes* verantwoordelijk zijn voor ziekte. Daarom zijn, met behulp van directe tellingen, huishoudens onderzocht op aanwezigheid van *Listeria* spp. In 101 (47%) van de 213 onderzochte huishoudens werd *L. monocytogenes* aangetoond. In vaatdoeken (37%) en in monsters rondom het afvoerputje in de douche (27%) werd dit micro-organisme het meest aangetroffen. De hoogste aantallen (ca 10^4 kve per object) werden gevonden in vaatdoeken en in afwasborstels. Uit een totaal van 871 monsters werden 132 *Listeria* spp. geïsoleerd. *L. innocua* (53%) en *L. monocytogenes* (41%) waren de meest voorkomende soorten. Hieruit blijkt dat persoonlijke en huishoudelijke hygiëne bij zouden kunnen dragen aan preventie van listeriose.

Dit proefschrift levert een belangrijke bijdrage aan de kennis die al beschikbaar is over het aantonen van *L. monocytogenes*, over het voorkomen van dit micro-organisme, en over het gedrag van deze ziekteverwekker in levensmiddelen.

SAMENVATTING

CURRICULUM VITAE

Rijkelt Richard Beumer werd geboren op 21 maart 1946 te Apeldoorn. Na het behalen van het diploma HBS-B aan het Christelijk Lyceum aldaar studeerde hij enige tijd medicijnen aan de Rijksuniversiteit Utrecht (RUU). Dit kon hem echter niet boeien, en hij ging in militaire dienst. Binnen een half jaar werd hij afgekeurd en twee maanden later, op 1 november 1966, trad hij in dienst van het toenmalige Instituut voor de Pluimveeteelt 'Het Spelderholt' te Beekbergen. Hij behaalde daar zijn analistendiploma's en begon de deeltijdstudie Biologie aan de RUU.

In 1971 kwam hij in dienst bij de Sectie levensmiddelenchemie en -microbiologie van de toenmalige Landbouwhogeschool te Wageningen. Zijn studie Biologie werd niet afgerond met een doctoraalexamen, maar in 1985 besloot een daartoe ingestelde commissie dat hij, op grond van zijn prestaties op het gebied van onderwijs en onderzoek, kon worden toegelaten tot de wetenschappelijke rangen. In dit proefschrift is het onderzoek beschreven dat hij, met zijn studenten, in de periode 1988-1996 heeft verricht.

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