ASPECTS AND OCCURRENCE OF *LISTERIA MONOCYTOGENES* IN MILK AND DAIRY PRODUCTS.

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ABSTRACT

This study covers six main areas. In the first area, the incidence of *Listeria* spp., in particular *L.monocytogenes* in raw and pasteurised milk and cheese samples purchased in Scotland was examined. It was shown that in cheese samples, *L.monocytogenes* was isolated from raw sheep mould-ripened cheese only and in raw cow, sheep and goat milk. It was not isolated from pasteurised cow milk.

In the second area, the use of impedance technology was used to study the effect of spent supernatant broths which had previously supported the growth of cheese ripening moulds on the growth of *Listeria* spp. This work was also carried out using cheese starter cultures. It was shown that some cheese ripening moulds could either partially inhibit or enhance the growth of *Listeria* spp. while one cheese starter culture partially inhibited the growth of *Listeria* spp.

In the third area, the manufacture and survival of *L.monocytogenes* in semi-soft and soft cheese with different cheese ripening moulds was studied. It was shown that *L.monocytogenes* can survive and grow readily in a Camembert cheese but not in a Blue-veined cheese. The previously shown *in vitro* effects on *L.monocytogenes* growth by cheese ripening moulds were not observed in the finished cheese products.

In the fourth area, the effect of heat treatment on *L.monocytogenes* was studied in sheep, cow and goat milk. It was shown that the sheep fat and some other unidentified factor in sheep skimmed milk had a protective effect on the survival of *L.monocytogenes* during heat treatments. It was shown that even

at low counts, *L.monocytogenes* could survive batch pasteurisation (62.8-65.6°C) but not HTST pasteurisation (71.1°C for 15 s).

In the fifth area, the effect of thermisation as a heat treatment for prolonging the keeping quality of milk used in cheese manufacture was studied. The survival of *L.monocytogenes* in this milk and cheese manufactured from it was also studied. It was shown that thermisation was not a suitable heat treatment for milk used in soft-cheese manufacture as *L.monocytogenes* survive thermisation and was found to subsequently survive in the final cheese.

The final area involved a study on the recovery and growth of *L.monocytogenes* and *L.innocua* in two *Listeria* enrichment broths (*Listeria* Enrichment Broth - LEB and *Listeria* Repair Broth - LRB). It was shown that using LEB, *L.innocua* could outgrow *L.monocytogenes* thus masking its presence using conventional isolation techniques. It was shown that LRB did not cause this effect.

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DECLARATION

I hereby declare that this thesis entitled " Aspects and Occurrence of *Listeria monocytogenes* in Milk and Dairy Products " is entirely my own work (excluding manufacture of Canadian cheddar cheese and Danish blue cheese, Chapter 7) and has not been accepted or submitted for any degree.

Signed

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LIST OF ABBREVIATIONS

cfu/ml	colony forming units per millilitre
dt	detection time
g	gram
>	greater than
h	hour
IMI	International Mycology Institute
<	less than
LEB	*Listeria* Enrichment Broth
LRB	*Listeria* Repair Broth
MEA	Malt Extract Agar
MRD	Maximum Recovery Diluent
MA	Milk Agar
MMB	Milk Marketing Board
min	minutes
mg	milligram
ml	millilitre
µl	microlitre
NCDO	National Collection of Dairy Organisms
NCTC	National Collection of Type Cultures
NA	Nutrient Agar
NB	Nutrient Broth
SITC	St Ivel Technical Centre
s	seconds
TSB	Tryptone Soya Broth

TSYEA	Tryptone Soya Yeast Extract Agar
TSYEB	Tryptone Soya Yeast Extract Broth

PAPERS PUBLISHED BY THE AUTHOR FROM WORK CONTAINED IN THESIS

Sutherland, A.D., Limond, A.M., MacDonald, F. and Hirst, D. (1993) Evaluation of the incubated plate count test for pasteurized milk. *Dairy Technology* **46**, 107-113.

MacDonald, F. and Sutherland, A.D. (1993) Effect of heat treatment on *Listeria monocytogenes* and Gram-negative bacteria in sheep, cow and goat milks. *Journal of Applied Bacteriology* **75**, 336-343.

MacDonald, F. and Sutherland, A.D. (1994) Important differences between the generation times of *Listeria monocytogenes* and *Listeria innocua* in two *Listeria* enrichment broths. *Journal of Dairy Research* **61**, 433-436.

Original Papers and Proceedings

Evaluation of the incubated plate count test for pasteurized milk

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Factors were examined which may influence bacterial psychrotroph counts obtained by the European Community's directed preincubation test for pasteurized milk. Studies to determine the best protocol for sampling milk indicated that the least counting error was obtained by taking at least five consecutive samples of milk. Increasing the number of replicate plates or making subsamples did not decrease counting error. When milk samples from five commercial dairies were examined over an 11 month period there was a significant difference ($p < 0.001$) in counts obtained over the period, but this did not seem to be a seasonal effect. The counts between dairies were not significantly different nor was the position in the pasteurization run from which the samples were taken significant. The proportion of samples which failed the test standard was 9.7% over the period. The bacterial flora isolated from plate count agar were identified; Gram-negative rods were the most common isolates. The spiral plater system was compared with the pour plate method in the preincubation test and results were found to be in close agreement, suggesting that the spiral plater could be used to improve the current official methods. Varying the time and temperature of sample preincubation was found to give significantly different results in plate counts. This suggested that preincubation conditons were critical and that variation in conditions even within the specified ranges of the test could significantly alter the bacterial psychrotroph counts obtained. Because of these results, the validity of the statutory test was questioned.

Introduction

Within the European Community (EC), under the EC Council Directive 85/397/EEC, last amended by Council Directive 89/662/EEC, pasteurized milk at the point of production is required to satisfy a microbiological standard based upon a preincubated plate count at 21°C, where the standard is not more than 100 000 per ml. This standard has now been incorporated into United Kingdom legislation. In England and Wales the relevant statutory instrument is The Milk (Special Designation) Regulations 1989, SI 2383.

The test is designed to assess the level of psychrotrophic bacteria in milk and therefore its likely keeping quality. Most psychrotrophic bacteria are Gram-negative rods which do not survive pasteurization and are present in milk as a result of postpasteurization contamination (Cousins, 1982). Because psychrotrophs can grow at low temperatures initial small numbers can multiply on cold storage and cause spoilage of the milk.

The EC test, which is based on preincubation methods such as those of Moseley (1975), involves preincubation of the milk at 6°C for five days to allow psychrotrophs to grow to detectable levels; a plate count is then performed at 21°C for 25 hours, a protocol under which psychrotrophs will outgrow other organisms (Oliveria and Parmelee, 1976; Griffiths *et al*, 1980).

There has been criticism of the test by the UK dairy industry and work carried out by the Milk Marketing Board of England and Wales has given some evidence of poor reproducibility (personal communication), a concern which has also been expressed by others (Anon, 1989). Undoubtedly, the long preincubation period requires strict control of incubation conditions.

This study therefore examined some factors which may give rise to variation in the psychrotroph count obtained by this test. Samples of commercially pasteurized milk were taken over a 10 month period to examine seasonal variation in psychrotroph counts,

Original paper.
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constituent bacterial flora and the effect of sample run position on psychrotroph counts obtained. Other work examined the effect on psychrotroph counts of incubating samples at various time/temperature combinations, including the extremes of the stipulated range allowed for in the time and temperature of sample incubation, ie, 120 ± 2 hours at 6° ± 0.5°C in an incubator controlled to ±0.2°C.

Materials and methods

Preliminary study on sampling protocol

The standard test methods do not indicate how many replicate samples should be taken from each pasteurization run. An initial experiment was designed to determine the between-pack and within-pack sample variation.

Eight packs each of fresh 1 pint cartons, 2 pint cartons and 1 pint bottles of pasteurized whole milk were purchased from a supermarket. The batch of samples was from the same shelf and of the same date code. Three separate batches were tested on three occasions.

Packs were aseptically transferred into 3 × 100 ml aliquots in sterile pots (Sterilin UK). The aliquots were incubated at 6°C for 120 hours and then plated in duplicate on milk agar plates (Oxoid). Plates were incubated at 21°C for 25 hours.

Seasonal survey of pasteurized milk

Unless otherwise stated, all materials and methods used for the preincubation of production milk were those specified in the *Official Journal of the EC* (Anon, 1991) under the Commission Decision 91/180/EEC.

All milk samples for the seasonal study were collected as whole units as produced. Samples were stored at 4°C overnight and transported in cold boxes at 4–6°C until analysis began. The pasteurization run conditions and sample collection protocol for each dairy were as outlined in Table 1.

Samples were plated on duplicate milk agar plates (Oxoid) using a spiral plater (Don Whitley Scientific Ltd, UK) as directed by BS4285:Section 2.3, Appendix A:1984 instead of the pour plate technique specified in the *Official Journal of the EC* (Anon, 1991).

TABLE 1
Pasteurization run time and sample time

Dairy	*Run time*	*Beginning sample*	*Middle sample*	*End sample*
1	0.700–20.00	07.10	12.30	19.45
2	08.00–14.00	08.00	11.00	14.00
3	06.00–14.50	11.45	13.00	14.05
4	10.00–15.00	10.05	11.00	13.00
5	08.00–13.00	08.00	10.30	12.30

This method was used for two reasons: (1) because the rapidity and cost effectiveness of the spiral plater system was necessary to process the number of samples involved in the study, and (2) this method facilitated the isolation and identification of the constituent microbial flora from the agar surface. The spiral plater has previously been shown (Donnelly *et al*, 1976; Jarvis *et al*, 1977; Kramer *et al*, 1979) to give results statistically comparable with the pour plate and other techniques.

A comparison of these two methods was also conducted in this study using pasteurized milk samples collected from commercial dairies. Three consecutive samples were taken from each of five dairies monthly for three months. These 45 samples were incubated at 6°C for five days and then were spread plated or spiral plated in duplicate.

Over an 11 month period, samples of pasteurized whole milk were collected as produced from the pasteurization run of five commercial dairies. Five consecutive samples were taken at the beginning, middle and end of the run, giving 15 samples/run in all. These samples were tested for psychrotrophic counts using the preincubation test to determine the seasonal variation in counts and fail rate of samples. From July each colony type which grew on plates was identified to genera and species level where possible.

Gram-negative isolates were identified using Enterotube II and Oxi/Ferm tube (Roche Products Ltd, UK) along with additional tests such as Gram-stain, catalase test, oxidase test. All genus and species identified here are as named in the strip tests. Non-sporing Gram-positive organisms were identified by following the identification schemes of MacFaddin (1980). Gram-positive spore-forming organisms were identified to species level using the API 50 strip test (Biomerieux). In addition, 200 µl duplicate volumes of milk were spread (after preincubation) directly onto Oxford and Mannitol-egg yolk–polymixin B agars (Oxoid) to assess the incidence and numbers of *Listeria* sp and *Bacillus cereus* respectively. *Listeria* were identified to species level by the methods described by McLauchlin (1989), which includes conventional carbohydrate fermentation tests. Samples from August onwards were also enriched in *Listeria* enrichment broth (Oxoid) and then streaked on Oxford agar using the International Dairy Federation method to confirm the absence of *Listeria* species in 25 ml of sample (Anon, 1990).

Experiments to compare the effects on psychrotroph counts of temperature and time variations

Aliquoted (100 ml) samples of milk purchased at retail outlets were incubated under various

time or temperature conditions to examine the effects on resultant psychrotroph counts. Psychrotroph counts in these retail samples were within the range determined in production samples taken in the seasonal study.

All incubators used in these studies were adjusted to within ±0.2°C of the required temperature. A 1 pint milk sample was also confirmed to hold a mean temperature of 6° ± 0.2°C over a five day incubation period by insertion of a squirrel temperature datalogger probe (Grant Instruments, UK) in the sample. Readings were taken as an average of 100 readings every 15 minutes for the period.

The specified permissible temperature range of sample incubation is 6° ± 0.5°C. In part A, samples were aliquoted and incubated at 4°, 6° and 8°C to determine the effect that such variation in temperature, which may be possible if incubators are not carefully regulated, would have on sample psychrotroph count. Five each of 1 pint cartons, 2 pint cartons and 1 pint bottles were collected on six separate occasions. Each package was aliquoted into 3 × 100 ml volumes and each aliquot was incubated at either 4°, 6° or 8° ± 0.2°C for five days. Also, a sample was removed and plated before incubation in duplicate for a time 0 psychrotroph count. After incubation, each aliquot was plated in duplicate and incubated at 21°C for 25 hours.

In part B, the effect on psychrotroph count obtained from aliquots of samples incubated at 5.5°, 6.0° and 6.5°C was examined to determine whether this constraint, ie, the specified temperature range of the test, was stringent enough. Five 1 pint cartons were collected on three separate occasions. Each package was aliquoted into 3 × 100 ml volumes and each aliquot incubated at either 5.5°, 6.0° or 6.5° ± 0.2°C for five days. After incubation each aliquot was plated in duplicate and incubated at 21°C for 25 hours.

Since the time of incubation for samples is specified (5 days ± 2 hours), the effects on psychrotroph counts of variations in incubation time were examined. Firstly, incubation periods of 5 days ± 6 hours, which could conceivably occur in busy laboratories, were examined. Secondly, incubations within the specified 5 days ± 2 hours were examined.

A. Five 1 pint milk cartons were collected from a supermarket, aliquoted into 3 × 100 ml volumes and held in an air incubator at 6°C for either 114, 120 or 126 hours (5 days ± 6 hours). Each aliquot was plated in duplicate. Plates were incubated at 21° for 25 hours and then counted. This protocol was repeated three times.

B. Five 1 pint milk cartons were collected from a supermarket, aliquoted into 3 × 100 ml volumes and held in an air incubator at 6°C for either 118, 120 or 122 hours (5 days ± 2 hours). This protocol was repeated on three separate occasions.

Statistical analysis

All data were $\log_{10}$ transformed and subjected to analysis of variance. Comparisons of the significance of results between groups of data were carried out by analysis of variance using the Genstat V program (Rothamsted Experimental Station, UK).

Results

Sampling protocol

An initial experiment was conducted to establish the relative between-pack and within-pack sampling variation of the test. Analysis showed the components of variance due to packs, aliquots and plates to be 1.255, 0.221 and 0.033 respectively—that is, the variance due to difference between packs was six times that due to aliquot differences, which in turn was six times that due to plating differences.

It was therefore concluded that to reduce test error it was sufficient to take only one sample per pack, but from as many packs as possible. It was, however, considered sensible to carry out duplicate platings.

Further work took account of these findings and five consecutive packs of milk were examined on duplicate agar plates.

Seasonal variation in bacterial count in pasteurized milk

Table 2 shows the mean $\log_{10}$ psychrotroph counts and Fig. 1 the failure rate of samples taken from March 1991 to January 1992. All data were cumulated, $\log_{10}$ transformed and subjected to analysis of variance. Counts were significantly different ($p < 0.001$) over the sampling period, reducing progressively (Table 2). The counts between dairies were not significantly different (Table 2) nor were they significantly different between sample position (beginning, middle and end). The number of samples which failed the standard over the 11 month period (Fig. 1) was 80/825 (9.7%).

The most common species of organisms identified from milk samples were psychrotrophic Gram-negative rods (76 isolates) including: *Pseudomonas* sp (50), *Acinetobacter* sp (10), *Achromobacter* sp (10) and *Flavobacteria* sp (6). However, *Bacillus* sp were also often isolated (53) and the other thermoduric isolates included *Micrococci* (5) and *Streptococci* (7). Other species included the coliform species *Escherichia coli*, Enterobacter and *Klebsiella* (24 isolates); *Shigella* (11 isolates), *Pasteurella/Moraxella* sp (16), *Corynebacteria* (8), *Aeromonas* (2), *Citrobacter* (2) and *Listeria* sp (8), which were presumably postpasteurization contaminants which survived (generally in low numbers) at 6°C.

Besides identifying the flora from milk agar plates, specific agars were also inoculated with milk samples to obtain direct counts for the psychrotrophic pathogenic organisms, *B cer-*

TABLE 2
Monthly psychrotroph counts from five dairies

Dairy	Pasteurization run position	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan
		(mean* log_{10} cfu/ml)										
1	Beginning	5.8	6.5	1.8	3.1	2.0	3.8	3.3	4.5	2.7	3.3	2.3
	Middle	1.3	6.4	1.7	3.1	2.9	2.3	4.4	3.5	3.0	2.9	3.1
	End	6.9	6.4	1.3	3.4	1.9	2.9	1.9	4.7	2.4	4.3	3.4
2	Beginning	4.4	3.7	3.3	3.9	3.4	2.5	2.5	3.6	3.3	3.9	1.4
	Middle	6.4	3.7	3.5	3.9	3.6	2.0	1.8	3.5	3.8	2.0	1.3
	End	4.0	3.7	3.4	3.8	2.7	2.4	1.5	3.3	3.4	1.9	1.4
3	Beginning	6.1	5.6	4.1	3.7	1.0	2.8	1.8	3.1	2.4	3.7	4.2
	Middle	8.1	5.9	4.0	5.3	1.9	2.7	1.5	3.5	3.7	2.7	2.5
	End	5.4	5.7	4.0	3.7	1.0	1.0	1.8	3.0	3.7	3.3	2.0
4	Beginning	5.8	3.9	5.1	2.1	3.1	2.4	6.2	ND	2.1	3.1	1.6
	Middle	6.1	3.7	5.3	2.1	3.0	2.3	1.9	ND	2.3	3.1	1.3
	End	5.9	2.8	5.8	2.4	3.0	2.3	2.1	ND	3.2	3.1	1.1
5	Beginning	6.9	4.2	2.8	2.8	3.5	2.7	3.9	4.2	4.0	1.9	2.5
	Middle	7.6	4.4	3.0	3.1	3.6	2.6	3.4	3.7	1.9	1.5	2.3
	End	7.0	4.1	2.8	2.6	3.3	2.2	3.1	4.0	2.1	1.8	2.4

Standard errors of the difference were 0.29 between months, 0.19 between dairies and 0.15 between run positions

* Mean psychrotroph count for 10 agar plates (five consecutive samples plated in duplicate).
ND = not determined.

eus and *Listeria monocytogenes*. The mean counts (cfu/ml) for *B cereus* are shown in Fig. 2. Over the 11 month sampling period 40/165 samples were found to have *B cereus* organisms present. *L monocytogenes* or other *Listeria* sp were not, however, recovered from direct plating on Oxford agar at any time. However, *Listeria* species including *L murrayi*, *L seeligeri* and *L innocua* were isolated in low numbers on eight occasions from milk agar used for bacterial counts.

Comparison of spiral plater with the pour plate method

The psychrotroph counts obtained for each sample (Table 3) by either the pour plate or spiral plate method were log_{10} transformed, correlated and subjected to analysis of variance. The psychrotroph counts obtained by each method correlated very well (r = 0.98) and by analysis of variance were found not to be significantly different at the 5% level. The difference between the mean log counts for the two methods was 0.08 ± 0.18. The cumulative counting error was also similar, the standard error of the mean being 0.13 and 0.12 for the pour plate and spiral plater methods respectively.

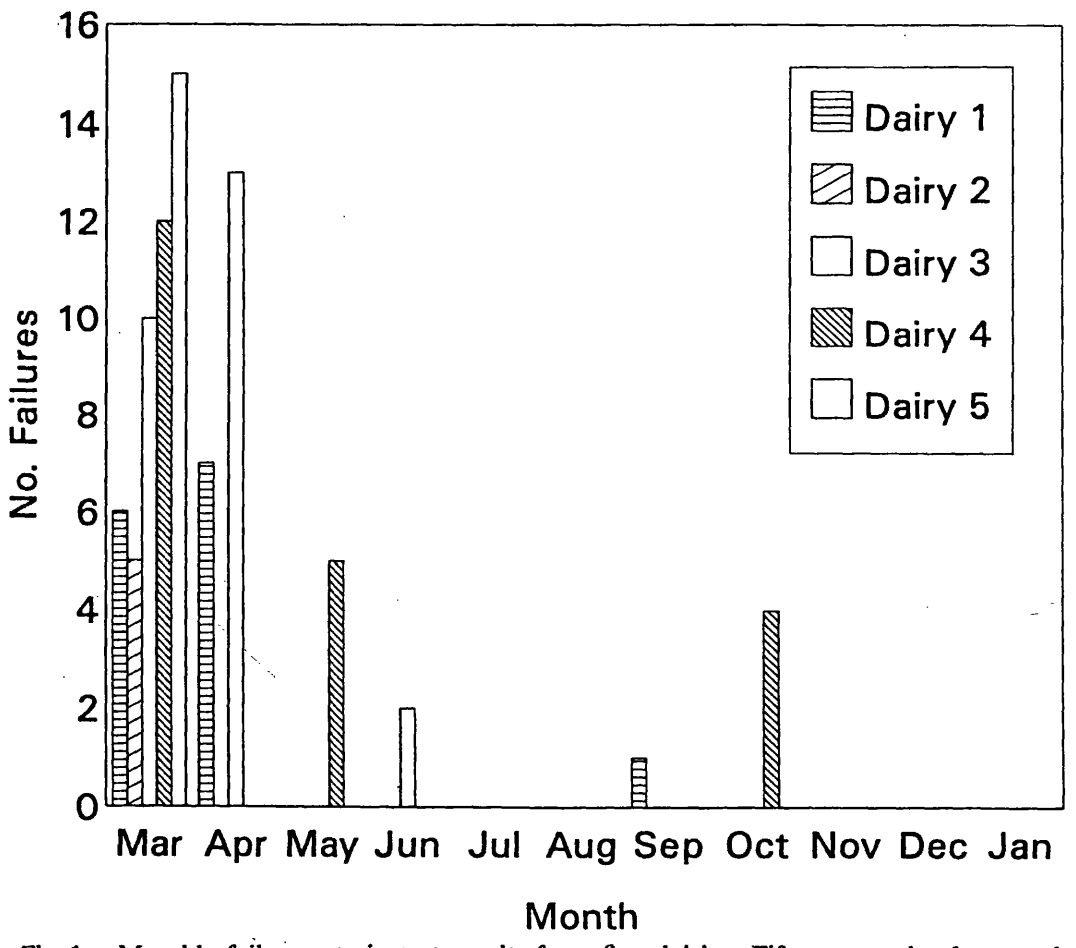

Fig. 1. Monthly failure rate in test results from five dairies. Fifteen samples from each dairy were tested monthly.

The effect of varying the incubation temperature on resultant psychrotroph count

(a) When comparing the psychrotroph counts obtained from samples of 1 pint cartons, 2 pint cartons and 1 pint bottles incubated at 4°C, 6°C or 8°C (Table 4), growth was significantly ($p < 0.001$) higher as the temperature of incubation increased (ie, counts at 4° < 6° < 8°C), except for 1 pint bottles where the psychrotroph count at 8°C was not significantly higher than the mean count at 6°C. The 1 pint bottle samples had significantly ($p < 0.001$) higher mean psychrotroph counts at time 0 than either 1 pint or 2 pint cartons and these counts increased at 4°C, 6°C and 8°C to be higher than those in corresponding 1 and 2 pint cartons. The mean log_{10} psychrotroph counts in the 1 pint bottle samples incubated at 6°C and 8°C for five days were high (6.7 ± 0.11 and 6.8 ± 0.11 respectively).

(b) The effect on psychrotroph count of variations in incubation temperature within the stipulated range of 6° ± 0.5°C was then examined. Volumes of milk were incubated at 5.5°, 6.0° or 6.5° ± 0.2°C for five days.

The psychrotroph counts obtained were log_{10} transformed (Table 5) and subjected to analysis of variance. Psychrotroph counts were significantly different ($p < 0.001$) at these temperatures, and the mean counts increased as the temperature of incubation

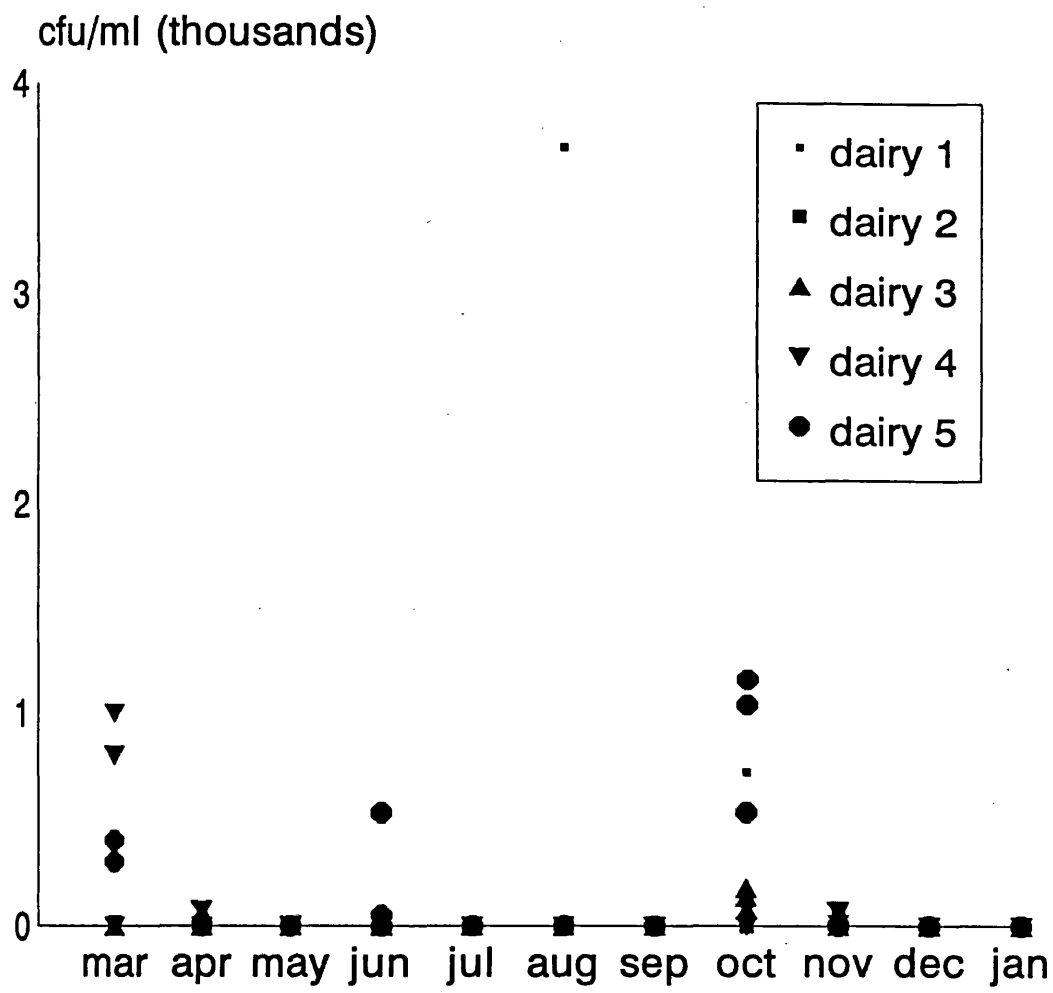

Fig. 2. Mean monthly counts of *Bacillus cereus* isolated from pasteurized milk from five dairies. The mean counts (colony forming units/ml) of five samples taken from the beginning, middle and end of the pasteurization run are shown.

rose from 5.5° to 6.0° and 6.5°C. The difference between the mean log counts at 5.5°C and 6.5°C was 1.3 ± 0.25.

The effect on psychrotroph count of altering the time of incubation

(a) Aliquots of milk were incubated at 6°C for 114, 120 or 126 hours, ie, ±6 hours of the 120 hours required in the standard method. The results were $\log_{10}$ transformed and subjected to analysis of variance. Differences in time of incubation of ±6 hours overall were found to give significant ($p < 0.001$) differences in the mean $\log_{10}$ psychrotroph counts (Table 6) obtained from samples. Counts in samples incubated for 126 hours were significantly ($p < 0.001$) higher than those in samples incubated for 114 or 120 hours. The mean counts at 114 and 120 hours were not, however, significantly different. The difference between mean log counts at 114 hours and 126 hours was 0.56 ± 0.11.

(b) Furthermore, aliquots of milk were incubated at 6°C for 118, 120 or 122 hours—

TABLE 3

Mean bacterial counts obtained from milk samples plated by the pour plate or spiral plate methods

No. of samples	*Log_{10} mean cfu/ml (± standard error)*	
	Pour plate	*Spiral plate*
90	2.95 (± 0.13)	3.03 (± 0.12)
Range of counts	0.9 – 6.0	1.4 – 5.81

Standard error of the difference between the methods was 0.18

TABLE 4

Mean* $\log_{10}$ bacterial count ($\log_{10}$ cfu/ml) in milk samples preincubated at 4°C, 6°C or 8°C for five days

	Sample		
Incubation	*1 pint carton*	*2 pint carton*	*1 pint bottle*
Start	2.3 (± 0.15)†	2.1 (± 0.15)	3.0 (± 0.15)
4°C	2.6 (± 0.15)	2.5 (± 0.16)	4.1 (± 0.11)
6°C	3.5 (± 0.15)	3.8 (± 0.16)	6.7 (± 0.11)
8°C	4.8 (± 0.15)	4.6 (± 0.16)	6.8 (± 0.11)

Standard error of the difference between temperatures for 1 pint cartons was 0.21, for 2 pint cartons 0.23 and for 1 pint bottles 0.16

* Mean of 60 plate counts.
† Figures in parentheses are the cumulated standard error of the mean.

TABLE 5

Mean* $\log_{10}$ bacterial count ($\log_{10}$ cfu/ml) in 1 pint milk samples preincubated at 5.5°C, 6.0°C or 6.5°C for five days

Incubation temperature	*Mean sample count (± SE mean)*
5.5°C	3.2 (± 0.18)
6.0°C	3.9 (± 0.18)
6.5°C	4.5 (± 0.18)

Standard error of the difference between temperatures was 0.25

* Mean of 30 plate counts.

TABLE 6

Mean ($\log_{10}$) bacterial counts ($\log_{10}$ cfu/ml) in samples incubated for varying periods

	Time of incubation (hours) at 6°C		
	114	*120*	*126*
Mean $\log_{10}$ cfu/ml (± SE)*	5.83 (± 0.08)	5.96 (± 0.08)	6.39 (± 0.08)

The standard error of the difference between times was 0.11

* Mean ± standard error of the mean of 15 samples plated in duplicate. Samples were collected on three separate occasions.

TABLE 7

Mean ($\log_{10}$) bacterial counts ($\log_{10}$ cfu/ml) in samples incubated for varying periods

	Time of incubation (hours) at 6°C		
	118	*120*	*122*
Mean $\log_{10}$ cfu/ml (± SE)*	5.07 (± 0.11)	4.98 (± 0.11)	5.38 (± 0.11)

The standard error of the difference between times was 0.15

* Mean ± standard error of the mean of 30 samples plated in duplicate. Samples were collected on three separate occasions.

that is, ±2 hours of the 120 hours' incubation required in the standard method. The psychrotrophic counts obtained were $\log_{10}$ transformed and subjected to analysis of variance and are summarized in Table 7. Overall differences in the temperature of incubation of ±2 hours were found to give significant ($p < 0.05$) differences in the mean psychrotroph counts (Table 7) obtained from

samples. Mean counts at 122 hours were significantly higher ($p < 0.05$) than at 120 and 118 hours. The mean counts at 118 and 120 hours were not, however, significantly different. The difference between mean log counts at 118 and 122 hours was 0.31 ± 0.16.

Discussion

Factors were examined which may influence the bacterial psychrotroph count obtained from the EC preincubation test.

The methods for the preincubation test do not specify how many samples are to be tested from each pasteurization run, but do recommend that counts are made from unopened milk containers or from 100 ml aliquots when necessary. A preliminary study therefore determined the influence of sample numbers, subsampling and replicate plating on milk samples. Increasing the number of subsamples made from each pack or the number of replicate plates did not significantly decrease the counting error. However, as the number of samples taken from the pasteurization run increased, the counting error decreased markedly. All subsequent sampling procedures therefore involved taking five consecutive samples of whole milk containers and preparing duplicate plates for psychrotroph counts after preincubation.

The influence of season and the position of the pasteurization run from which the samples were taken was examined over an 11 month period. Psychrotroph counts obtained from samples taken at five dairies varied significantly over the sampling period. In the first month (March 1991) 48/75 samples had psychrotroph counts greater than 100 000 cfu/ml. This failure rate dropped progressively thereafter until from June there were few failures from any dairy. The reason for this drop in failure rate is unknown but did not seem to be a seasonal effect since failure rate dropped in a linear manner and did not increase again in January or February 1992. The dairies participating in the study were aware of the purpose for sampling. Overall, there was no significant difference in psychrotroph counts obtained from each dairy.

Samples were taken from the beginning, middle and end of each pasteurization run. Although some variation was apparent there was no significant difference in the psychrotroph counts obtained from each run position. This suggested that sampling from only one run position should be adequate for testing purposes.

The constituent microflora in samples was identified monthly from June 1991. As expected from the findings of others (see review of Cousins, 1982), Gram-negative rods were the dominant psychrotrophs isolated and were presumably postpasteurization contaminants. *Bacillus* species were also frequently isolated. The spores of these species can survive pasteurization and psychrotrophic species have also been identified in both raw and pasteurized milk (Grosskopf and Harper, 1969; Chung and Cannon, 1971; Shehata and Collins, 1971; Coghill and Juffs, 1979). *B cereus* was isolated on six occasions from plate count agar and on 22 occasions from *B cereus* selective agar (Oxoid). Counts of *B cereus* from selective agar ranged from $1.0–3.7 \times 10^3$ cfu/ml in positive samples. These milk samples were incubated at 6°C for five days before plating; a reasonable simulation of storage in a household refrigerator. *B cereus* counts in foods implicated in food poisoning outbreaks have been reported as low as 1×10^3 cfu/ml (Giannella and Brasile, 1979; Kramer and Gilbert, 1989). Dairy products have been implicated in food poisonings due to *B cereus*, although rather infrequently (Vlad and Vlad, 1972; McSwiggan *et al*, 1975; Schmitt *et al*, 1976). That *B cereus* was more frequently isolated on selective agar than from plate count agar suggests that selective agars are beneficial when examining milk samples for this organism.

In contrast, *Listeria* species (other than *L monocytogenes*) were isolated on eight occasions from plate count agar, whereas no *Listeria* were recovered from direct plating on Oxford agar, or on samples which were first enriched in *Listeria* enrichment broth and then streaked on Oxford agar. This suggests that *Listeria* species present in milk in small numbers may not always be detectable by enrichment/selective procedures such as those outlined by the International Dairy Federation (Anon, 1990). Reasons for this may include the presence of damaged or stressed bacterial cells in samples. Busch and Donnelly (1992) have recently described a resuscitation broth and procedure which allows the recovery of heat shocked *Listeria* cells in greater numbers than could be recovered by conventional enrichment.

When compared, the psychrotroph counts obtained by using a spiral plater correlated well with those obtained by the pour plate method. The counting error was also very similar.

These findings show that the spiral plating method is comparable with the pour plate method and are in agreement with those of Jarvis *et al* (1977). Published official regulations and methods do not always keep pace with the latest technology available and discussed in the literature. The spiral plater is a rapid, less laborious and inexpensive system to run than other conventional plating methods. Since it gives comparable results to spread plating, this system should be considered for inclusion in the EC microbiological counting methods for dairy products.

Because the temperature of incubation (6°C) is selective for the growth of psychrotrophs and since the incubation period of the

test is long (5 days) temperature control within the preincubation period is likely to be critical.

The effect on psychrotroph counts obtained by the preincubation test by altering the temperature of incubation was assessed. The statutory method stipulates that incubators must be set at 6° ± 0.2°C and that samples must not vary by more than 6° ± 0.5°C temperature.

Samples of milk incubated at 4°, 6° or 8°C were found generally to give significantly higher psychrotroph counts as the temperature of incubation increased.

It is apparent from Table 4 that variation in the temperature of incubation of 2°C either side of the stipulated 6°C has a significant effect on the psychrotroph counts obtained. Therefore, there is obviously a potentially significant source of error in the test if incubators are not carefully regulated.

The influence of altering the temperature of incubation within the statutory range of the test was also examined. When volumes of milk were incubated at 5.5°, 6.0° and 6.5° ± 0.2°C for five days the psychrotroph counts were again found to increase as the temperature of incubation rose. This implies that the stipulated range from temperature control is not sufficiently rigorous to give reproducible results between two samples incubated at the extremes of the range. It is probably practically impossible to maintain a busy laboratory incubator within a stricter range of temperatures, however, and this must call into question the validity of the EC preincubation test.

The effect of altering the time of incubation on the preincubation test was also considered. The stipulated range for the test is 120 ± 2 hours.

In the first instance the effect of incubating samples for 120 ± 6 hours was examined and found to result in highly significant differences in psychrotroph count. It was apparent, then, that strict attention must be paid to the duration of incubation. Psychrotroph counts were also examined after incubation of sample aliquots at 120 ± 2 hours. Psychrotroph counts were again found to be significantly different when comparing aliquots incubated within this range. This also implies that the current stipulated range is insufficiently rigorous to give comparable results between two samples incubated at the extremes of the range.

The influence of incubating samples at the extremes of the range of temperature and time of incubation was found to give statistically significant differences in psychrotroph counts; the effect on the overall failure rate of pasteurized milk samples was not determined, however, and so remains unknown.

It is apparent from this study that the conditions for the preincubation test must necessarily be rigorously controlled because of the long incubation period at 6°C. It is uncertain whether stricter control than the regulations already impose would be practically possible. This therefore questions the validity of the EC preincubation test as a reproducible statutory implement.

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Effect of heat treatment on *Listeria monocytogenes* and Gram-negative bacteria in sheep, cow and goat milks

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F. MACDONALD AND A.D. SUTHERLAND. 1993. Sheep milk, compared with cow and goat milk, had a protective effect on Gram-negative bacteria and *Listeria* spp. heated at 65°C in a test-tube method. This effect was not solely due to fat content as cow milk artificially reconstituted to 10% homologous fat was not as protective. *Listeria monocytogenes* in whole sheep, cow and goat milks at an inoculum level of 1×10^6 cfu ml^{-1} was heated at 68°C for 15 s in the plate pasteurizer and survival was only detected in whole sheep milk after heating. Whole sheep, cow and goat milks containing high levels of *L. monocytogenes* (1×10^6 cfu ml^{-1}) could not survive the current HTST plate pasteurization protocol.

INTRODUCTION

Past outbreaks of listeriosis have focused attention on the presence of *Listeria monocytogenes* and the ability of this organism to survive pasteurization in milk and dairy products. An outbreak of listeriosis in Massachusetts during 1983, which caused 14 deaths, was associated with consumption of pasteurized whole or 2% fat milk. Although *L. monocytogenes* was not isolated from the plant environment it was found in the raw milk from one supplier (Fleming *et al.* 1985). This suggested either that *L. monocytogenes* survived pasteurization or that the high temperature short time (HTST) pasteurization (71·7°C/15 s) was not carried out correctly. A 1985 outbreak in California, which resulted in 48 deaths, was linked to consumption of a Mexican-style fresh cheese. It is believed that the pasteurized milk used to produce the cheese was contaminated with raw milk containing *L. monocytogenes* (Linnan *et al.* 1988). This outbreak highlighted the ability of this pathogen to survive in a processed dairy product where post-pasteurization contamination occurs.

The heat resistance of *L. monocytogenes* has been in question for a number of years. Bearns and Girard (1958) who used the 'holding technique' of pasteurization (61·7°C for 35 min), showed that viable *L. monocytogenes* could survive in cow milk whenever the initial number was 5×10^4 cfu ml^{-1} or greater. This was also confirmed by Donnelly *et al.* (1987) who used this method, but with another 'sealed tube' method they also showed that with an inoculum of 10^6–10^7 cfu ml^{-1}, *L. monocytogenes* could not survive under the same conditions of heating.

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Several workers have shown that *L. monocytogenes* cannot survive HTST pasteurization (71·7°C/15 s) in cow milk (Bradshaw *et al.* 1985; Beckers *et al.* 1987; Lovett *et al.* 1990).

Many workers have published findings on the thermal resistance of *L. monocytogenes* (conflicting or otherwise) but mainly with cow (bovine) whole or skimmed milk or in broths. No published work of this type has used sheep (ovine) or goat (caprine) milks. A single case of listeric meningitis in a healthy individual in the UK in 1986 due to the consumption of goat milk soft cheese contaminated with *L. monocytogenes* (Azadian *et al.* 1989) indicated that all milk types, not just cow (bovine), could possibly produce potentially hazardous cheeses or other dairy products. Sheep, cow and goat milks are all used in the production of cheese and need to be investigated. *Listeria monocytogenes* occurs more frequently in cheese compared with many other processed dairy products as the milk used is often unpasteurized or given minimal heat treatment (64·4°C/16 s) (Johnson *et al.* 1990).

Listeria monocytogenes has also been shown to survive when added to milk used to make Camembert (Ryser and Marth 1987a), Cheddar (Ryser and Marth 1987b), blue (Papageorgiou and Marth 1989) and semi-hard cheese (Dominguez *et al.* 1987).

Many types of cheese are produced from a variety of milk types or from a combination of two or more (e.g. Spanish Manchego cheese). All have varying levels of fat content, bacterial flora and heat treatments. *Listeria monocytogenes* can grow between 1 and 45°C (Seeliger and Jones 1986) and can readily survive and grow at refrigeration temperatures (2–8°C) at which most dairy products are maintained or ripened. Psychrotrophic Gram-negative bacilli

are common contaminants of raw milk (Bramley and McKinnon 1990) which can grow at low temperatures and cause spoilage. The best means of ensuring that pathogens such as *L. monocytogenes* and important spoilage organisms are not transferred to cheese and other dairy products is adequate pasteurization. Sheep milk generally has a higher fat content (5·8–9·1% weight) than cow (3·2–5·1% weight) or goat milk (2·8–6·5% weight) (Tamine *et al.* 1991) and it is a concern that this higher fat content may protect bacteria from the effects of heating. To examine this possibility this work investigated the survival of *L. monocytogenes* and Gram-negative psychrotrophic spoilage bacteria in heat-treated sheep, cow and goat milks with varying fat levels.

MATERIALS AND METHODS

Bacterial strains

Three *L. monocytogenes* strains were used in this study, SITC (St Ivel Technical Centre, UK) 12/1A, SITC 404/2 and NCTC (National Collection of Type Cultures, London, UK) 7973 and one *L. innocua* strain, SITC 236/2/8. The four psychrotrophic Gram-negative bacilli strains used in this study are all common milk isolates. These included *Pseudomonas fluorescens* NCDO 2085 (National Collection of Dairy Organisms changed to NCFD (National Collection of Food Bacteria), Shinfield, Nr Reading, Berks, UK), *Citrobacter freundii* GTE 022, *Acinetobacter lwoffii* GTE 024 and *Klebsiella ozoaenae* GTE 019 (all from the Hannah Research Institute collection). The *Listeria* strains were grown in Tryptone Soya Broth (Oxoid) and all other strains were grown in Nutrient Broth (Oxoid) at 37°C for 24 h to prepare inocula.

Milk

Raw cow and goat milks were obtained on site from the Hannah Research Institute and raw sheep milk was collected from a local sheep farm and maintained at between 2 and 8°C until arrival at the Institute. The milk was dispensed into sterile containers (1 l) and centrifuged at 56 *g* (Mistral 6L). The milk was separated from the top cream layer by suction and then filtered through Whatman No. 1, 15 cm filter paper.

The fat percentage of whole and skimmed milk was estimated by the Gerber method (Anon. 1989). The skimmed milk and fat were homogenized (Silverson Emulsifier, Chesham, Bucks, UK) until the fat was dispersed in the milk. Fat was added to the milk so that the resulting suspension contained 0, 5 or 10% fat (w/v). In some experiments, where indicated, whole milk with the original fat content (without separation or resuspension) was used.

Temperature experiments

Water bath. Milk was dispensed in 10 ml volumes into duplicate sterile glass test-tubes with lids. One hundred μl of 24 h cultures were added to each tube and mixed. The samples were placed in a water-bath at 65(±0·2), 68(±0·2) or 72°C(±0·2) for 0, 15, 30 or 45 min. The samples took up to 2 min 15 s (at 72°C) to equilibrate to the required temperature and were cooled as quickly as possible after heating by immersion in an ice-bath. All trials were repeated on three separate occasions.

Plate pasteurizer. An APV Junior heat exchanger system (Junior Paraflow, APV Baker, Derby, UK) modified to heat samples up to 140°C was used. It was set variously at 68, 70, 72 and 74°C with a holding time of 15 s. This was maintained by the insertion of a tube of appropriate length in the holding section with a product flow of 90 l h^{-1}. Ten l of milk were inoculated with *Listeria* sp. to give 1×10^6 cfu ml^{-1}. The milk was pasteurized at 68, 70, 72 and 74°C with a holding time of 15 s and cooled as quickly as possible by immersion in an ice-bath. All trials were repeated on two separate occasions.

Bacterial enumeration

Total plate counts were made by spread plating 500 μl samples and appropriate dilutions (Maximum Recovery Diluent; Oxoid) in duplicate at each heating interval on either Milk Agar (Oxoid) for Gram-negative bacilli or on Oxford Selective Agar (Oxoid) for *Listeria* spp. after incubation at 37°C for 48 h.

Bacterial confirmation

Colonies of *Listeria* spp. isolated from milk samples after heat treatment were identified as grey colonies surrounded by black zones caused by aesculin hydrolysis on Oxford agar. Selected colonies were confirmed on the five separate occasions of testing as *Listeria* spp. by the following criteria: Gram-positive, non-sporing, oxidative-negative, catalase-positive bacilli showing 'tumbling' motility in wet mounts after growth in Tryptone Soya Broth (Oxoid) supplemented with 6% Yeast Extract (Oxoid) incubated at 21°C for 6 h.

Colonies which were presumed to be of one of the four Gram-negative bacilli used for inocula were biochemically confirmed from the Gram stain and the oxidase test. Oxidase-positive isolates (i.e. *Ps. fluorescens*) were identified by Oxi/Ferm tubes (Roche) and all oxidase-negative isolates (i.e. *Ac. lwoffii*, *Cit. freundii* and *Kl. ozoaenae*) were identified with Enterotube II (Roche).

Bacterial strain	cfu ml^{-1} after (min) 0	15	30	45
No fat added to skim				
Pseudomonas fluorescens NCDO 2085	$1{\cdot}1 \times 10^7$	<1	<1	<1
Citrobacter freundii GTE 022	$1{\cdot}0 \times 10^7$	<1	<1	<1
Acinetobacter lwoffii GTE 024	$7{\cdot}1 \times 10^6$	<1	<1	<1
Klebsiella ozoaenae GTE 019	$3{\cdot}4 \times 10^6$	<1	<1	<1
5% fat added to skim				
Pseudomonas fluorescens NCDO 2085	$>5 \times 10^7$	$8{\cdot}5 \times 10^3$	<1	<1
Citrobacter freundii GTE 022	$2{\cdot}8 \times 10^7$	$1{\cdot}8 \times 10^3$	<1	<1
Acinetobacter lwoffii GTE 024	$2{\cdot}1 \times 10^7$	$5{\cdot}7 \times 10^3$	<1	<1
Klebsiella ozoaenae GTE 019	$2{\cdot}0 \times 10^7$	$7{\cdot}6 \times 10^2$	<1	<1
10% fat added to skim				
Pseudomonas fluorescens NCDO 2085	$9{\cdot}5 \times 10^6$	$2{\cdot}2 \times 10^4$	<1	<1
Citrobacter freundii GTE 022	$1{\cdot}1 \times 10^7$	$9{\cdot}1 \times 10^2$	<1	<1
Acinetobacter lwoffii GTE 024	$1{\cdot}0 \times 10^7$	$5{\cdot}7 \times 10^3$	<1	<1
Klebsiella ozoaenae GTE 019	$1{\cdot}0 \times 10^7$	$4{\cdot}2 \times 10^3$	<1	<1

<1 = No bacteria recovered, detection limit was 1 cfu ml^{-1}.

Table 1 Survival of Gram-negative bacteria at 65°C for various times as a function of fat content of sheep milk

RESULTS

Gram-negative bacteria

Trials using inocula of 10^6–10^7 cfu ml^{-1} of four strains of Gram-negative bacteria heated in cow, goat and sheep milks containing 0, 5 or 10% fat and the test-tube method showed that no organisms were recoverable within 15 min from all cow and goat milk samples (results not shown), while heating for 30 min at 65°C was required for sheep milk samples containing 5 and 10% fat before there were no organisms recoverable (Table 1). This indicated that sheep milk samples containing 5 or 10% fat had a protective effect on the survival of the organisms during heating.

The survival of Gram-negative bacteria was further studied in whole sheep milk (6·58% fat) and the test-tube method heated at 65, 68 and 72°C for 15 or 30 min (Table 2). It was shown that these bacteria could survive for 15 min even at 72°C but were not recoverable after heating for 30 min.

Further trials with skim cow and sheep milks made up to 10% fat content with either homologous or heterologous fat

Temperature (°C)	Bacterial strain	cfu ml^{-1} after (min) 0	15	30
65	*Pseudomonas fluorescens* NCDO 2085	$7{\cdot}0 \times 10^6$	$6{\cdot}4 \times 10^3$	<1
	Citrobacter freundii GTE 022	$8{\cdot}6 \times 10^6$	$8{\cdot}1 \times 10^3$	<1
	Acinetobacter lwoffii GTE 024	$6{\cdot}8 \times 10^6$	$1{\cdot}0 \times 10^3$	<1
	Klebsiella ozoaenae GTE 019	$3{\cdot}4 \times 10^6$	$9{\cdot}2 \times 10^3$	<1
68	*Pseudomonas fluorescens* NCDO 2085	$7{\cdot}0 \times 10^6$	$2{\cdot}6 \times 10^3$	<1
	Citrobacter freundii GTE 022	$8{\cdot}6 \times 10^6$	$5{\cdot}0 \times 10^2$	<1
	Acinetobacter lwoffii GTE 024	$6{\cdot}8 \times 10^6$	$3{\cdot}8 \times 10^2$	<1
	Klebsiella ozoaenae GTE 019	$3{\cdot}4 \times 10^6$	$4{\cdot}9 \times 10^2$	<1
72	*Pseudomonas fluorescens* NCDO 2085	$7{\cdot}0 \times 10^6$	$1{\cdot}9 \times 10^3$	<1
	Citrobacter freundii GTE 022	$8{\cdot}6 \times 10^6$	$1{\cdot}9 \times 10^2$	<1
	Acinetobacter lwoffii GTE 024	$6{\cdot}8 \times 10^6$	$2{\cdot}6 \times 10^2$	<1
	Klebsiella ozoaenae GTE 019	$3{\cdot}4 \times 10^6$	<1	<1

<1 = No bacteria recovered, detection limit was 1 cfu ml^{-1}.

Table 2 Survival of Gram-negative bacterial strains at 65, 68 and 72°C for various times in whole sheep milk (6·58% fat)

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Table 3 Survival of Gram-negative bacteria at 65°C for 15 min in sheep or cow skimmed milk supplemented with sheep or cow milk fat

Bacterial strain	Mean cfu ml^{-1} after (min) 0	15
Sheep skimmed milk + 10% sheep fat		
Pseudomonas fluorescens NCDO 2085	$7{\cdot}3 \times 10^6$	$5{\cdot}5 \times 10^3$
Citrobacter freundii GTE 022	$9{\cdot}3 \times 10^6$	$8{\cdot}8 \times 10^3$
Acinetobacter lwoffii GTE 024	$5{\cdot}0 \times 10^6$	$6{\cdot}8 \times 10^3$
Klebsiella ozoaenae GTE 019	$3{\cdot}6 \times 10^6$	$8{\cdot}6 \times 10^3$
Sheep skimmed milk + 10% cow fat		
Pseudomonas fluorescens NCDO 2085	$1{\cdot}9 \times 10^6$	$1{\cdot}7 \times 10^4$
Citrobacter freundii GTE 022	$5{\cdot}5 \times 10^6$	$1{\cdot}3 \times 10^4$
Acinetobacter lwoffii GTE 024	$2{\cdot}3 \times 10^6$	$7{\cdot}8 \times 10^3$
Klebsiella ozoaenae GTE 019	$3{\cdot}1 \times 10^6$	$1{\cdot}0 \times 10^4$
Cow skimmed milk + 10% cow fat		
Pseudomonas fluorescens NCDO 2085	$1{\cdot}6 \times 10^7$	<1
Citrobacter freundii GTE 022	$9{\cdot}3 \times 10^6$	<1
Acinetobacter lwoffii GTE 024	$2{\cdot}5 \times 10^6$	<1
Klebsiella ozoaenae GTE 019	$4{\cdot}6 \times 10^6$	<1
Cow skimmed milk + 10% sheep fat		
Pseudomonas fluorescens NCDO 2085	$1{\cdot}1 \times 10^7$	$4{\cdot}9 \times 10^3$
Citrobacter freundii GTE 022	$9{\cdot}8 \times 10^6$	$1{\cdot}5 \times 10^3$
Acinetobacter lwoffii GTE 024	$5{\cdot}4 \times 10^6$	$6{\cdot}5 \times 10^3$
Klebsiella ozoaenae GTE 019	$4{\cdot}5 \times 10^6$	$1{\cdot}5 \times 10^4$

<1 = No bacteria recovered, detection limit was 1 cfu ml^{-1}.

(Table 3) showed that sheep fat protected Gram-negative bacteria from heating at 65°C for 15 min in cow and sheep skim milks. However, cow fat added to cow skim milk did not protect the organism from heating, but cow fat added to sheep skim was protective.

Listeria species

Trials with inocula of 10^6–10^7 cfu ml^{-1} of four *Listeria* strains in cow and sheep milk containing 0, 5 or 10% fat were conducted using the test-tube method at 65°C.

Table 4 Survival of *Listeria* strains at 65°C for various times as a function of fat content of cow milk

Bacterial strain	cfu ml^{-1} after (min)* 0	15	30	45
No fat added to skim				
L. monocytogenes SITC 12/1A	$3{\cdot}00 \times 10^6$	<1	<1	<1
L. monocytogenes SITC 404/2	$2{\cdot}80 \times 10^6$	<1	<1	<1
L. monocytogenes NCTC 7973	$6{\cdot}15 \times 10^6$	$1{\cdot}80 \times 10^2$	<1	<1
L. innocua SITC 236/2/8	$6{\cdot}60 \times 10^6$	85	<1	<1
5% fat added to skim				
L. monocytogenes SITC 12/1A	$3{\cdot}37 \times 10^6$	55	<1	<1
L. monocytogenes SITC 404/2	$2{\cdot}17 \times 10^6$	$1{\cdot}50 \times 10^2$	<1	<1
L. monocytogenes NCTC 7973	$6{\cdot}40 \times 10^6$	$5{\cdot}35 \times 10^2$	<1	<1
L. innocua SITC 236/2/8	$3{\cdot}82 \times 10^6$	$2{\cdot}43 \times 10^2$	<1	<1
10% fat added to skim				
L. monocytogenes SITC 12/1A	$2{\cdot}38 \times 10^6$	$4{\cdot}53 \times 10^3$	$3{\cdot}68 \times 10^2$	<1
L. monocytogenes SITC 404/2	$4{\cdot}76 \times 10^6$	$3{\cdot}83 \times 10^3$	14	<1
L. monocytogenes NCTC 7973	$5{\cdot}18 \times 10^6$	$1{\cdot}57 \times 10^3$	55	<1
L. innocua SITC 236/2/8	$8{\cdot}14 \times 10^6$	$8{\cdot}14 \times 10^3$	$4{\cdot}12 \times 10^2$	<1

* Mean of 12 replicate counts taken on three occasions.
<1 = No bacteria recovered, detection limit was 1 cfu ml^{-1}.

Table 5 Survival of *Listeria* strains at 65°C for various times as a function of fat content of sheep milk

Bacterial strain	cfu ml^{-1} after (min)*			
	0	15	30	45
No fat added to skim				
L. monocytogenes SITC 12/1A	$7{\cdot}93 \times 10^6$	$6{\cdot}92 \times 10^3$	62·5	<1
L. monocytogenes SITC 404/2	$6{\cdot}55 \times 10^6$	$3{\cdot}18 \times 10^3$	14·0	<1
L. monocytogenes NCTC 7973	$1{\cdot}59 \times 10^7$	$1{\cdot}61 \times 10^4$	$4{\cdot}51 \times 10^2$	<1
L. innocua SITC 236/2/8	$1{\cdot}61 \times 10^7$	$4{\cdot}28 \times 10^4$	97·0	<1
5% fat added to skim				
L. monocytogenes SITC 12/1A	$1{\cdot}01 \times 10^7$	$1{\cdot}05 \times 10^4$	$4{\cdot}22 \times 10^3$	$2{\cdot}13 \times 10^3$
L. monocytogenes SITC 404/2	$7{\cdot}90 \times 10^6$	$2{\cdot}44 \times 10^3$	$4{\cdot}09 \times 10^2$	28
L. monocytogenes NCTC 7973	$1{\cdot}32 \times 10^7$	$2{\cdot}89 \times 10^3$	$2{\cdot}53 \times 10^3$	<1
L. innocua SITC 236/2/8	$1{\cdot}63 \times 10^7$	$1{\cdot}83 \times 10^4$	$2{\cdot}72 \times 10^3$	$2{\cdot}01 \times 10^2$
10% fat added to skim				
L. monocytogenes SITC 12/1A	$1{\cdot}12 \times 10^7$	$6{\cdot}29 \times 10^3$	$4{\cdot}58 \times 10^3$	$1{\cdot}65 \times 10^3$
L. monocytogenes SITC 404/2	$6{\cdot}83 \times 10^6$	$6{\cdot}75 \times 10^3$	$8{\cdot}16 \times 10^2$	$4{\cdot}99 \times 10^2$
L. monocytogenes NCTC 7973	$5{\cdot}16 \times 10^6$	$1{\cdot}10 \times 10^3$	$1{\cdot}32 \times 10^2$	77
L. innocua SITC 236/2/8	$1{\cdot}87 \times 10^7$	$6{\cdot}04 \times 10^3$	$3{\cdot}43 \times 10^3$	$9{\cdot}79 \times 10^2$

* Mean of 12 replicate counts taken on three occasions.
<1 = No bacteria recovered, detection limit was 1 cfu ml^{-1}.

Results showed that no organisms were recoverable within 45 min for all cow milk samples (Table 4) and skim sheep milk (no fat), while sheep milk samples containing 5 and 10% fat still contained viable organisms after 45 min (Table 5).

The survival of *L. monocytogenes* (SITC 12/1A) was further studied in sheep milk containing 10% fat. With the test-tube method varying levels of inoculum were heated at 65°C for up to 45 min. A starting inoculum as low as $8{\cdot}9 \times 10^2$ cfu ml^{-1} survived for 30 min but no organisms were recovered after heating for 45 min (Table 6).

Further trials with 10% homologous or heterologous fat (Table 7) in skim cow and sheep milks showed sheep fat to be protective to all *Listeria* strains after heating at 65°C for 45 min in both cow and sheep skim milks. Cow fat added to cow skim milk, however, did not protect these bacteria from heating after 30 min at 65°C, but cow fat added to sheep skim was protective, although to a lesser extent than sheep fat added to sheep milk.

The survival of *Listeria* spp. in sheep milk was further studied in whole sheep milk (7% fat) and the test-tube method heated at 65, 68 and 72°C for 15, 30 or 45 min (Table 8). It was shown that at 68°C all bacterial strains could survive for 30 min but no organisms were recovered within 15 min at 72°C.

However, when whole sheep milk containing 1×10^6 cfu ml^{-1} of *L. monocytogenes* (SITC 12/1A) was heated in a plate pasteurizer at 68, 70, 72 and 74°C for 15 s only 10 cfu

Table 6 Survival of *Listeria monocytogenes* (SITC 12/1A) at 65°C for various times at 10% fat content of sheep milk

Start inoculum level (cfu ml^{-1})	cfu ml^{-1} after (min)*			
	0	15	30	45
Neat	$4{\cdot}44 \times 10^6$	$2{\cdot}64 \times 10^4$	$3{\cdot}67 \times 10^3$	$1{\cdot}04 \times 10^3$
1/5 D	$2{\cdot}70 \times 10^6$	$1{\cdot}54 \times 10^4$	$9{\cdot}03 \times 10^2$	$6{\cdot}46 \times 10^2$
1/10 D	$1{\cdot}63 \times 10^6$	$1{\cdot}25 \times 10^4$	$1{\cdot}72 \times 10^3$	$8{\cdot}47 \times 10^2$
1/50 D	$5{\cdot}70 \times 10^4$	$2{\cdot}31 \times 10^4$	$2{\cdot}78 \times 10^2$	$6{\cdot}39 \times 10^2$
1/100 D	$6{\cdot}83 \times 10^3$	$7{\cdot}99 \times 10^2$	$7{\cdot}70 \times 10^2$	$4{\cdot}17 \times 10^2$
1/500 D	$1{\cdot}50 \times 10^3$	$4{\cdot}86 \times 10^2$	$4{\cdot}51 \times 10^2$	$4{\cdot}33 \times 10^2$
1/1000 D	$1{\cdot}00 \times 10^3$	$2{\cdot}01 \times 10^2$	$1{\cdot}18 \times 10^2$	$8{\cdot}33 \times 10^1$
1/5000 D	$8{\cdot}90 \times 10^2$	14	7	<1

* Mean of eight replicates taken on two occasions.
D, Dilution using maximum recovery diluent.
<1 = No bacteria recovered, detection limit was 1 cfu ml^{-1}.

Table 7 Survival of *Listeria* strains at 65°C for various times with 10% fat (sheep or cow) in sheep and cow milks

Bacterial strain	cfu ml^{-1} after (min)*			
	0	15	30	45
10% sheep fat added to sheep skim milk				
L. monocytogenes SITC 12/1A	$2{\cdot}25 \times 10^7$	$1{\cdot}93 \times 10^3$	$1{\cdot}32 \times 10^3$	$2{\cdot}50 \times 10^2$
L. monocytogenes SITC 404/2	$1{\cdot}08 \times 10^7$	$2{\cdot}45 \times 10^3$	$1{\cdot}01 \times 10^3$	$6{\cdot}50 \times 10^2$
L. monocytogenes NCTC 7973	$1{\cdot}50 \times 10^7$	$2{\cdot}77 \times 10^3$	$9{\cdot}50 \times 10^3$	$3{\cdot}11 \times 10^2$
L. innocua SITC 236/2/8	$1{\cdot}93 \times 10^7$	$5{\cdot}12 \times 10^3$	$2{\cdot}65 \times 10^3$	$4{\cdot}55 \times 10^2$
10% cow fat added to sheep skim milk				
L. monocytogenes SITC 12/1A	$2{\cdot}25 \times 10^7$	$1{\cdot}53 \times 10^3$	$1{\cdot}85 \times 10^2$	20
L. monocytogenes SITC 404/2	$2{\cdot}49 \times 10^7$	$1{\cdot}37 \times 10^3$	$4{\cdot}22 \times 10^2$	95
L. monocytogenes NCTC 7973	$2{\cdot}18 \times 10^7$	$3{\cdot}11 \times 10^3$	$3{\cdot}34 \times 10^2$	65
L. innocua SITC 236/2/8	$2{\cdot}11 \times 10^7$	$2{\cdot}50 \times 10^3$	$4{\cdot}75 \times 10^2$	75
10% cow fat added to cow skim milk				
L. monocytogenes SITC 12/1A	$3{\cdot}25 \times 10^7$	$1{\cdot}75 \times 10^3$	$1{\cdot}55 \times 10^2$	<1
L. monocytogenes SITC 404/2	$1{\cdot}58 \times 10^7$	$1{\cdot}20 \times 10^3$	$2{\cdot}12 \times 10^2$	<1
L. monocytogenes NCTC 7973	$5{\cdot}25 \times 10^7$	$3{\cdot}16 \times 10^3$	59	<1
L. innocua SITC 236/2/8	$4{\cdot}50 \times 10^7$	$3{\cdot}74 \times 10^3$	$1{\cdot}73 \times 10^2$	<1
10% sheep fat added to cow skim milk				
L. monocytogenes SITC 12/1A	$2{\cdot}08 \times 10^7$	$7{\cdot}66 \times 10^2$	$3{\cdot}00 \times 10^2$	$1{\cdot}40 \times 10^2$
L. monocytogenes SITC 404/2	$1{\cdot}26 \times 10^7$	$3{\cdot}75 \times 10^3$	$4{\cdot}78 \times 10^2$	$1{\cdot}94 \times 10^2$
L. monocytogenes NCTC 7973	$1{\cdot}75 \times 10^7$	$3{\cdot}02 \times 10^3$	$2{\cdot}25 \times 10^2$	85
L. innocua SITC 236/2/8	$2{\cdot}75 \times 10^7$	$4{\cdot}50 \times 10^3$	$5{\cdot}72 \times 10^2$	$2{\cdot}11 \times 10^2$

* Mean of 12 replicates taken on three occasions.
<1 = No bacteria recovered, detection limit was 1 cfu ml^{-1}.

ml^{-1} survived at 68°C and at all other temperatures no organisms were recovered. The same inoculum of *L. monocytogenes* did not survive in whole cow (3·27% fat) or goat (2·85%) milk when heated at 68, 70, 72 and 74°C for 15 s on the plate pasteurizer (results not shown).

DISCUSSION

Sheep milk was found to have factors which protected bacteria against heating. The protective effect seemed to be specific to sheep milk and not solely due to the fat percent-

Table 8 Numbers of *Listeria* surviving heat treatment at 65, 68 and 72°C for various times in whole sheep milk (7% fat)

Temperature (°C)	Bacterial strain	cfu ml^{-1} after (min)*			
		0	15	30	45
65	*L. monocytogenes* SITC 12/1A	$2{\cdot}48 \times 10^7$	$2{\cdot}13 \times 10^4$	$3{\cdot}00 \times 10^3$	$1{\cdot}60 \times 10^2$
	L. monocytogenes SITC 404/2	$2{\cdot}65 \times 10^7$	$2{\cdot}00 \times 10^4$	$6{\cdot}70 \times 10^2$	$1{\cdot}50 \times 10^2$
	L. monocytogenes NCTC 7973	$2{\cdot}09 \times 10^7$	$9{\cdot}25 \times 10^3$	$4{\cdot}20 \times 10^2$	$1{\cdot}35 \times 10^2$
	L. innocua SITC 236/2/8	$3{\cdot}25 \times 10^7$	$6{\cdot}00 \times 10^4$	$3{\cdot}00 \times 10^3$	$5{\cdot}00 \times 10^2$
68	*L. monocytogenes* SITC 12/1A	$2{\cdot}69 \times 10^7$	$2{\cdot}00 \times 10^3$	$1{\cdot}80 \times 10^2$	<1
	L. monocytogenes SITC 404/2	$2{\cdot}23 \times 10^7$	$1{\cdot}72 \times 10^3$	$5{\cdot}40 \times 10^2$	<1
	L. monocytogenes NCTC 7973	$2{\cdot}94 \times 10^7$	$1{\cdot}00 \times 10^3$	$1{\cdot}80 \times 10^2$	<1
	L. innocua SITC 236/2/8	$2{\cdot}51 \times 10^7$	$1{\cdot}50 \times 10^3$	$2{\cdot}40 \times 10^2$	<1
72	*L. monocytogenes* SITC 12/1A	$2{\cdot}76 \times 10^7$	<1	<1	<1
	L. monocytogenes SITC 404/2	$2{\cdot}14 \times 10^7$	<1	<1	<1
	L. monocytogenes NCTC 7973	$3{\cdot}06 \times 10^7$	<1	<1	<1
	L. innocua SITC 236/2/8	$2{\cdot}97 \times 10^7$	<1	<1	<1

* Mean of 12 replicates taken on three occasions.
<1 = No bacteria recovered, detection limit was 1 cfu ml^{-1}.

age as cow and goat milks artificially made up to 10% homologous fat content were not found to protect Gram-negative bacteria against heating. Cow milk artificially made up to 10% homologous fat content also did not protect strains of *L. monocytogenes* or *L. innocua* against heating.

Listeria innocua was used in this study to determine if any effect due to the heat treatment given to the milk type and combinations could be shown between non-human pathogenic (i.e. *L. innocua*) and human pathogenic (*L. monocytogenes*) *Listeria* species. The results show that all the heat treatments gave similar results on the survival of all the *Listeria* strains and species examined.

The (UK) Milk (Special Designation) Regulations 1989 state that, for pasteurized whole cow milk, the heat treatment should be not less than 62·8°C and not more than 65·6°C for at least 30 min (batch pasteurization) or not less than 71·7°C for at least 15 s (plate pasteurization) or an equivalent process. The results for the heating of sheep milk showed that even a small level of inoculum of *L. monocytogenes* (i.e. $8{\cdot}9 \times 10^2$ cfu ml^{-1}) could survive the batch pasteurization conditions specified in the above regulation. The results obtained using the plate pasteurizer indicate that it was far more efficient at heat killing than the test-tube method.

Bradshaw *et al.* (1985) stated that *L. monocytogenes* strain Scott A in raw milk had a *D*-value at 68·9°C of 3 s and at 71·7°C of 0·09 s. The results we obtained with the plate pasteurizer at 72°C were in agreement with theirs in that *L. monocytogenes* did not survive pasteurization at 72°C for 15 s, but their findings suggest that *L. monocytogenes* would have survived at 68°C in whole goat, cow and sheep milks. Our findings showed that *L. monocytogenes* was not detectable after heating at 68°C in cow and goat milks but survival did occur in sheep milk. This again indicated that *L. monocytogenes* (SITC 12/1A) has a greater heat resistance in whole sheep milk rather than whole cow or goat milk.

The Gram-negative bacteria used in this study were less heat-resistant at 65°C in whole cow and sheep milk than the four *Listeria* strains used (three *L. monocytogenes* and one *L. innocua*) but could survive heating at 72°C for 15 min in the test-tube method while the four *Listeria* strains could not. The reasons for this finding are unclear as previous work has indicated that *Listeria* is generally more heat-resistant than other bacteria (Mackey and Bratchell 1989).

The protective factor(s) present in sheep skim milk and sheep milk fat were demonstrated with both Gram-negative and Gram-positive bacteria.

Since psychrotrophic Gram-negative organisms can survive heating at 72°C for 15 min and *Listeria* spp. can survive heating at 65°C for 45 min it would therefore be necessary to pasteurize whole sheep milk at 72°C for 30 min with the vat (test-tube) method. Even high levels of *L. monocytogenes* (1×10^6 cfu ml^{-1}) in whole sheep, cow and goat milks could not survive the current HTST plate pasteurization protocol.

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Important differences between the generation times of *Listeria monocytogenes* and *List. innocua* in two *Listeria* enrichment broths

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Listeria monocytogenes is the only species in the genus *Listeria* that is recognized as pathogenic to man. Most species of the genus *Listeria* have been isolated from raw cows' milk (Lovett *et al.* 1987; Harvey & Gilmour 1992; Rea *et al.* 1992), the most frequently isolated being *List. innocua* and *List. monocytogenes.*

Since *Listeria* spp. are generally present in low numbers, their isolation from milk is usually successful only after enrichment procedures. Harvey & Gilmour (1992), for instance, never isolated *List. monocytogenes* by direct plating of 176 raw milk samples but isolated *List. monocytogenes* and *List. innocua* by enrichment procedures from 15·3 and 10·2% of samples respectively.

From our unpublished results, we have found that the incidence of *List. monocytogenes* in raw ewes', cows' and goats' milks was considerably lower than that of *List. innocua*. On no occasion were both species isolated from the same sample after enrichment broth culture. The presence of *Listeria* spp. in samples cultured in enrichment broths is usually shown after incubation by streaking a loopful of the broth on to a selective agar and selecting five presumptive *Listeria* colonies for further identification (International Dairy Federation, 1990). It occurred to us that if there were differences in growth rate of the two species in enrichment broths then it would be likely that only the dominant species would be identified after culture.

Isolation of *List. innocua* from a food sample is not considered hazardous, but if it outgrew co-cultured *List. monocytogenes* during enrichment culture procedures then it would be masking the presence of this potentially dangerous pathogen.

This study was designed to determine whether *List. innocua* could outgrow *List. monocytogenes* when cultured either separately or in mixed culture in two selective *Listeria* enrichment broths. Generation times for each *Listeria* spp. were calculated by impedance techniques and confirmed for one strain of each species by conventional plating methods.

MATERIALS AND METHODS

Listeria *cultures*

List. monocytogenes strains used were Scott A, L519, L527, L533, L560, L580 (isolated from raw cows' milk, Hannah Research Institute Collection), and *List. innocua* strains were NCTC 11288, (National Culture Type Collection), L510, L523, L534, L553 and L585 (isolated from raw cows' milk, Hannah Research Institute Collection). *List. monocytogenes* and *List. innocua* strains used in this study were grown from single colonies on Oxford agar (Oxoid, Basingstoke, RG24 0PW) in tryptone soya broth (TSB, Oxoid) at 37 °C for 18 h before being used as inocula for growth studies.

Listeria *enrichment broths*

The *Listeria* enrichment broths used in this study were *Listeria* enrichment broth (LEB, Oxoid) and *Listeria* resuscitation broth (LRB, Busch & Donnelly 1992). The selective supplements for *Listeria* growth were added to the LEB before incubation while supplements for LRB were added 5 h after the start of incubation to assist in the revival of stressed bacteria.

Determination of generation times

Impedance studies. A Bactometer, Model M64 with disposable module cassettes (BioMérieux UK Ltd, Basingstoke RG22 6HY) was used in this study. Module wells were filled with 1 ml TSB, LEB or LRB. These wells were inoculated in duplicate with 10 μl of either a 10^{-6} or a 10^{-4} dilution of a single strain of *List. monocytogenes* or *List. innocua* TSB culture. The cassettes were incubated at 30 °C for 50 h and capacitance was monitored throughout. In this study *List. monocytogenes* (Scott A) and *List. innocua* (NCTC 7973) were repeated on three separate occasions while the remaining strains were each tested on one occasion.

By recording the differences in detection times between the 100-fold dilutions of the initial inoculum, the generation time (t_g) for each *Listeria* sp. was calculated using the following equation (Firstenberg-Eden & Eden, 1984).

$$t_g = (\Delta IDT \times \log 2)/(\log N_1 - \log N_2),$$

where IDT is the initial detection time, $\log N_1$ is the log count of the lower dilution of inoculum and $\log N_2$ is the log count of the higher dilution of inoculum. Since $(\log N_1 - \log N_2) = 2$ and $\log 2 = 0{\cdot}301$,

$$t_g = 0{\cdot}15 \times \Delta IDT.$$

Plate count study. Volumes (30 ml) of TSB, LEB and LRB were inoculated with 30 μl of a 10^{-5} dilution of a TSB culture of *List. monocytogenes* (Scott A) or *List. innocua* (NCTC 11288) and incubated at 30 °C for 24 h. Every 2 h undiluted samples (500 μl) or appropriate dilutions were spread plated or, for later samples, spiral plated (Spiral Plater model D; Don Whitley, Shipley BD17 7SE) on to duplicate Oxford agar plates (International Dairy Federation, 1990). All plates were incubated at 37 °C for 48 h.

The generation time of microbial populations was determined using conventional plate counts and calculating the growth rate constant (k) from the exponential growth curves determined for *List. monocytogenes* and *List. innocua*.

$$k = 2{\cdot}303(\log Z - \log Z_0)/(t - t_0),$$

where Z and Z_0 were the cfu/ml at the measuring time t and initial time t_0 respectively. From this, t_g was calculated by $k = \ln 2/t_g$, whence $t_g = 0{\cdot}693/k$, expressed in hours.

Fate of List. monocytogenes *and* List. innocua *in mixed culture*

List. monocytogenes (Scott A) and *List. innocua* (NCTC 11288) were inoculated into 225 ml LEB to yield $\sim$ 10 cfu/ml of each species. The enrichment broth was incubated at 30 °C for 48 h and then a loopful streaked on to duplicate Oxford agar plates (International Dairy Federation, 1990). After incubation at 37 °C for 24 h, eight presumptive *Listeria* colonies were selected from Oxford agar and grown on tryptone soya agar supplemented with yeast extract (TSYEA, 60 g/l, Oxoid) for

Table 1. *Mean generation times (min) of* Listeria monocytogenes *and* List. innocua *in broths using impedance methods*†

Bacterial strain	Broth: Tryptone soya	Broth: *Listeria* enrichment	Broth: *Listeria* resuscitation
List. monocytogenes			
Scott A	44·4	78·6	61·5
L519	41·0	68·4	55·4
L527	33·8	62·6	51·8
L533	50·0	72·9	50·4
L560	40·5	67·1	53·1
L580	38·3	68·0	55·8
Grand mean	42·9	71·9	50·2
SEM	1·1	3·6	2·5
List. innocua			
NCTC 11288	43·7	52·5	53·1
L510	41·4	53·1	60·8
L523	53·6	56·3	49·2
L534	41·9	59·9	43·7
L553	38·3	56·7	46·8
L585	39·2	55·4	50·4
Grand mean	43·0	54·8	51·2
SEM	1·8	1·5	2·1

† Strains Scott A and NCTC 11288 were tested in duplicate on three separate occasions. All other strains were tested once in duplicate.

identification. All colonies were examined for morphology, catalase, oxidase and motility at 21 °C. Differentiation between the species was achieved using the API *Listeria* test strip (BioMérieux).

RESULTS AND DISCUSSION

The generation times between strains of *List. monocytogenes* and *List. innocua* in TSB (non-selective broth) and LRB were not significantly different (Table 1). The generation times for *List. monocytogenes* and *List. innocua* strains in LEB were significantly different ($P < 0{\cdot}001$), with those for *List. innocua* strains being faster (Table 1). This result indicated that *List. innocua* should outgrow *List. monocytogenes* in LEB. This prompted a further experiment wherein *List. monocytogenes* and *List. innocua* were inoculated in equal numbers into a single LEB and incubated at 30 °C for 48 h. It was found that all of eight randomly selected colonies isolated from streaked plates of incubated enrichment broth were identified as *List. innocua* (API *Listeria* code 7510). *List. monocytogenes* was not detected.

Similar growth patterns were obtained for *List. monocytogenes* (Scott A) and *List. innocua* (NCTC 11288) using conventional plating and impedance methods, but in all cases calculated generation times were faster using the impedance method (Table 2). The differences in generation times between the two methods may be due to impedance techniques relying on the measurement of metabolic changes, whereas the plate count method depends on the production of a visible biomass. With both *List. monocytogenes* and *List. innocua* strains the generation times overall were faster in TSB than in LRB, and slowest in LEB. The differences in generation times of species were probably due to differences in susceptibility to inhibition by enrichment broth components and incubation procedures since generation times were the same for both species in TSB. Duh & Schaffner (1993) have shown that *List. innocua* grows faster

Table 2. *Generation times (min) of* Listeria monocytogenes *and* List. innocua *in broths using impedance and plate count methods*

(Values are means for three experiments)

	Impedance method		Plate count method	
Broths	*List. monocytogenes*	*List. innocua*	*List. monocytogenes*	*List. innocua*
Tryptone soya	44·4	43·7	54·5	50·1
Listeria enrichment	78·6	52·5	94·6	77·9
Listeria resuscitation	61·5	53·1	79·3	75·9

than *List. monocytogenes* at temperatures < 42 °C, but no significant differences in the growth rates of these species were shown using TSB at 30 °C.

It was found that when both *List. monocytogenes* and *List. innocua* were grown together in LEB, *List. innocua* outgrew *List. monocytogenes*. The implication is that a food sample containing both of these species may well be considered free from *List. monocytogenes* since it would not be readily recovered from LEB. At present, the International Dairy Federation (1990) recommends the use of LEB for detection of *List. monocytogenes* in milk and milk products by enrichment procedures. Using LEB as a selective enrichment broth for the isolation of *List. monocytogenes* from a food sample may result in this organism not being recovered if significant levels of *List. innocua* are also present.

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CHAPTER 1

LITERATURE REVIEW

1.1 HISTORY OF THE GENUS *LISTERIA*

The organism *Listeria monocytogenes* (*L.monocytogenes*) was first described by Murray *et al.* in 1926 and named *Bacterium monocytogenes* due to characteristic monocytosis found in the liver of infected rabbits and guinea pigs. In 1927, Pirie isolated the bacterium from the liver of gerbils and he named it *Listerella hepatolytica* (Gray and Killinger, 1966). It was later shown that the name *Listerella* had already been given to a Mycetozoan so was changed to its present name *Listeria* by Pirie in 1940 (Pirie, 1940). Gill was credited with the first isolation of *L.monocytogenes* from domesticated farm animals in 1929 and Nyfeldt with the first confirmed report of listeric infection in man in the same year (Gray and Killinger, 1966).

Until 1961 *L.monocytogenes* was the only recognised species in the genus *Listeria* until *L.denitrificans*, *L.grayi* and *L.murrayi* were added in 1961, 1966 and 1971, respectively (Seelinger and Jones, 1986). In 1974, Ivanov proposed that all serovar 5 strains showing strong β-haemolysis be placed in a seperate species, *L.bulgarica* (Farber and Peterkin, 1991) which was officially named *L.ivanovii* in 1984 (Seelinger *et al.*, 1984). Nonpathogenic strains of *L.monocytogenes* belonging to serovar 6 were rocognised as a new species *L.innocua* in 1979 and *L.welshimeri* and *L.seeligeri* were added to the genus in 1983 (Seelinger and Jones, 1986).

The genus *Listeria* was named by Pirie after Lord Lister, an English

surgeon. The type species of the genus is *L.monocytogenes*. *L.innocua* was named after the latin *innocuus* meaning harmless as it has been shown to be nonpathogenic to man. *L.denitrificans* was named due to its ability to reduce nitrates. The remaining species in the genus are named after predominant people in the field of microbiology, namely *L.welshimeri* in honour of Herbert J. Welshimer (the American bacteriologist), *L.seeligeri* in honour of Heinz P. R. Seeliger (the German bacteriologist), *L.ivanovii* in honour of Ivan Ivanov (the Bulgarian microbiologist), *L.grayi* in honour of Mitchell L. Gray (the American microbiologist) known for his work in the field of listeriosis and *L.murrayi* in honour of Edward G. D. Murray, co-discoverer of *L.monocytogenes* (Seeliger and Jones, 1986).

Sporadic cases of human listeriosis have occurred over the last 60 years, mainly from workers in contact with animals infected with *L.monocytogenes*, but it was not until 1957 that foodborne listeriosis was first suspected in three outbreaks (between 1949-1957) involving raw milk (Ryser and Marth, 1991). In 1976, *L.monocytogenes* was still not recognised as a foodborne pathogen by the World Health Organisation (Anon, 1976). This discrepancy was soon highlighted in the 1980s when several outbreaks of listeriosis were associated with the consumption of food containing *L.monocytogenes* (James *et al.*, 1985; Linnan *et al.*, 1988; Azadian *et al.*, 1989 and Bille, 1990).

1.2 CURRENT TAXONOMY OF THE GENUS *LISTERIA*

In the 9th and current edition of Bergey's Manual of Systematic Bacteriology, the genus *Listeria* is listed as containing 8 species with 5 as accepted species, which are *L.monocytogenes, L.innocua, L.welshimeri, L.seeligeri*

and *L.ivanovii*. The 3 remaining species are *L.grayi, L.murrayi* and *L.denitrificans* and are all listed as *Species Incertae Sedis*, meaning being of uncertain ground to be included as accepted species of the genus *Listeria* at this present time (Seeliger and Jones, 1986).

From numerical taxonomic, biochemical, serological, morphological and nucleic acid studies, it was concluded that *L.denitrificans* was quite different from the other *Listeria* spp. and constituted a single monospecific genus (Welshimer and Meredith, 1971; Stuart and Pease, 1972; Stuart and Welshimer, 1973; Stuart and Welshimer, 1974; Jones, 1975; Jones, 1975; Wilkinson and Jones, 1975; Collins *et al.*, 1983; Fiedler and Seger, 1983; Fiedler *et al.*, 1984; Jones *et al.*, 1986 and Espaze *et al.*, 1986). Consequently *L.denitrificans* has now been transferred to a new genus *Jonesia* and renamed *J.denitrificans* (Rocourt *et al.*, 1987).

Stuart and Welshimeri (1974) proposed that *L.grayi* and *L.murrayi* be transferred to a new genus *Murraya*, and classified as *M.grayi subsp grayi* and *M.grayi subsp murrayi* respectively. Molecular biological studies have also confirmed that *L.grayi* and *L.murrayi* should be reclassified as one species, *L.grayi* (Rocourt *et al.*, 1992). However, from current taxonomic studies, it has been shown that *L.grayi* and *L.murrayi*, although having resulted from a different line of descent, are closely related to the remaining *Listeria* species and so should remain as part of the genus *Listeria* (Jones, 1992).

In 1992, Boerlin *et al.* showed that *L.ivanovii* could be divided into two main genomic groups using multilocus enzyme electrophoresis. These two

genomic groups represent two subspecies, *L.ivanovii* subsp. *ivanovii* and *L.ivanovii* subsp. *londoniensis* subsp. nov.

The present taxonomic position of the genus *Listeria* is that it now consists of 6 species, namely *L.monocytogenes*, *L.innocua*, *L.welshimeri*, *L.seeligeri*, *L.ivanovii* and *L.grayi*, with *L.denitrificans* excluded from the genus and transferred to a new genus (Jones, 1992). The genus *Listeria* is related to the genus *Bronchothrix*, with both these genera occupying a position between *Lactobacillus* and *Bacillus* and are more distantly related to *Streptococcus*, *Lactococcus*, *Enterococcus*, *Staphylococcus*, *Kurthia*, *Gemella* and *Erysipelothrix* (Seeliger and Jones, 1986).

1.3 MORPHOLOGY OF THE GENUS *LISTERIA*

The species of the genus *Listeria* are Gram positive, regular short rods 0.4-0.5 μm in diameter and 0.5-2.0 μm in length with rounded ends. Some cells can be curved and can also occur singly in short chains or in a V form or in groups lying parallel along the axis.

Smith and Metzger (1962) showed the presence of a mucopolysaccharide capsule in *L.monocytogenes*. This was not shown by Seeliger and Bockemûhl (1968) and the organism is now listed in the latest edition of Bergey's Manual of Systematic Bacteriology (Seeliger and Jones, 1986) as not forming a capsule. Spores are also not formed.

Cells are motile by a few peritrichous flagella when cultured at 20-25^{o}C, exhibiting characteristic tumbling motility (Gray and Killinger, 1966). At 37^{o}C, flagella development is so poor that organisms appear non-motile (Peel *et*

al., 1988). Motility can be demonstrated using the Hanging Drop technique (see section 2.2.9) or by inoculation into semi-solid Nutrient Agar (NA) in a modified Craigie or U tube and incubating at room temperature. Progress of motile bacteria can be followed by a clouding of the medium in the outer agar around the Craigie tube or just below the surface in the uninoculated arm of the U tube.

These organisms are aerobic and facultative anaerobes. Stab inoculation in glucose semi-solid medium results in growth along the stab after 24 h with maximal growth occurring 3-5 mm below the surface of the medium in an umbrella-like pattern (Seeliger and Jones, 1986).

24-48 hour colonies on Nutrient Agar are 0.5-1.5mm in diameter, round, translucent with a dew-drop appearance. They are also low, convex with a finely textured surface and entire margin (Seeliger and Jones, 1986). The colonies appear bluish gray by normal illumination and a characteristic blue-green sheen is produced by obliquely transmitted light (Henry, 1933).

Some species of the genus *Listeria* are β-haemolytic on Blood Agar (BA). Strains of *L.ivanovii* produce wide zones of haemolysis (Seeliger and Jones, 1986) while *L.monocytogenes* and *L.seeligeri* produce narrow zones which in some cases can only be detected by removal of the colony. A weak or doubtful β-haemolytic reaction can be highlighted by use of the CAMP test (Christie *et al.*, 1944).

The optimum growth temperature is between 30°C and 37°C but the growth range is between 0.5-45.0°C (Seeliger and Jones, 1986). *Listeria* spp. are therefore psychrotrophic. A factor which is significant in the contamination

of cold-stored foods. Optimum growth occurs at neutral or slightly alkaline pH. Some strains can grow at pH 9.6 but all usually die at a pH lower than 5.5. Growth can also occur in Nutrient Broth supplemented with up to 10% (w/v) sodium chloride (Seeliger and Jones, 1986).

These organisms are catalase positive, oxidase negative, Methyl-Red positive and Voges-Proskauer positive (Seeliger and Jones, 1986).

There are at least 16 serovars of the *Listeria* genus based on 14 heat-stable somatic (O) antigens and 4 heat-labile flagellar (H) antigens. This system does not include the serologically different species of *L.grayi*. With the exception of *L.ivanovii* (serovar 5 strains only), there is no correlation between between serovar and species as *L.monocytogenes* strains exhibiting the antigenic composition of serovars 1/2b, 4c and 4d are also found in strains of *L.seeligeri* (Seeliger and Jones, 1986).

Studies on carbohydrate fermentations by the genus *Listeria* have shown that all the species can utilise glucose resulting in production of mainly L(+)-lactic acid. Acid but no gas is produced from a number of other sugars. The ability to ferment certain sugars has been used to differentiate between the *Listeria* species. These fermentation reactions and some others are used to differentiate between the *Listeria* spp as outlined in Table 1 (Jones, 1990).

1.4 PATHOGENICITY OF THE GENUS *LISTERIA*

A number of *Listeria* species have been shown to cause infection in man and animals, these include *L.monocytogenes, L.ivanovii* and *L.seeligeri*. The four

remaining species have not been shown to cause infection. In humans, only three infections with *L.ivanovii* and one with *L.seeligeri* have been reported (McLauchlin, 1987). Rocourt and Seeliger (1985) showed that 98% of strains in humans and 87% of strains in animals causing disease were identified as *L.monocytogenes.* Between the period 1967 to 1985, McLauchlin (1990) showed that all the 722 strains from cases of human listeriosis sent to The (PHLS) Division of Microbiology Reagents and Quality Control at Colindale (London) were identified as *L.monocytogenes.*

L.monocytogenes appears to be a normal resident of the intestinal tract in humans. This may partially explain why antibodies to *Listeria* spp. are common in healthy people. The number of human carriers of *L.monocytogenes* as assessed by the examination of faecal samples ranged from 0.5 - 69.2% (and as high as 91.7% in one study involving 12 female laboratory technicians) (Ralovich, 1984). Thus, because of the high rate of clinically healthy carriers, the presence of *L.monocytogenes* in the faeces is not necessarily an indication of infection. The ability of *L.monocytogenes* to cause infection depends on host susceptibility (this is discussed later in section 1.5), infectious dose and virulence factors of the organism. The infectious dose for this pathogen is still presently unknown. Protein p60 and listeriolysin O are recognised as the main virulence factors associated with *L.monocytogenes.* Protein p60 promotes adhesion and penetration into mammalian cells by inducing phagocytosis. Inside the phagocytes listeriolysin O lyses the membrane-bound phagocytic vacuole. Once released onto the cell cytoplasm, *L.monocytogenes* reacts with the host-cell microfilaments and becomes enveloped in a thick coat of F-actin,

which facilitates penetration into adjacent cells where listeriolysin O lyses of the membrane bound phagocytic vacuole is repeated (Jones, 1990).

Infection by *L.monocytogenes* can occur by oral, ocular, cutaneous, respiratory or urogenital routes. Modes of transmission from animals to humans include ingestion of contaminated food and contact with infected animals (Pearson and Marth, 1990). Ingestion of *L.monocytogenes* is followed by penetration of the gastrointestinal tract by the bacterium (Ryser and Marth, 1991).

1.5 HUMAN LISTERIOSIS

The majority of human cases of listeriosis occur in individuals who have an underlying condition which leads to suppression of their T cell mediated immunity. These conditions include immunosupression, pregnancy and extremes of age (very young or elderly). Listeriosis can also however occur in apparently healthy young adults (Gellin and Broome, 1989). The clinical features of listeriosis range from influenza-like illness to meningitis and meningoencephalitis. The incubation period for listeriosis varies from 1 to 70 days (Jones, 1990).

Infection in pregnant woman usually leads to only influenza-like illness, with fever, headache and myalgia. It is rarely life threatening to the mother. Infection of the fetus can however result in premature labour, septic abortion or delivery of an acutely ill infant. Two clinical forms of neonatal listeriosis have been described and these are early or late onset neonatal listeriosis. Early onset neonatal listeriosis occurs in infants infected in utero and is apparent at

birth or within a few days of life and develops as a disease known as *granulomatosis infantisepticum*. It is characterised by pneumonia, septicaemia and disseminated absesses. Mortality rate is high at 40 - 50% (Gellin and Broome, 1989). Late onset neonatal listeriosis manifests itself several days or weeks after birth of the infant, with clinical manifestation of infection likely to be meningitis. Mortality rate is lower than early onset neonatal listeriosis at about 25% (Gellin and Broome, 1989).

In non-pregnant, immunosuppressed individuals, meningitis or bacteraemia/septicaemia are the most common clinical features. The world estimated mortality rate for listeriosis is about 30% (Gellin and Broome, 1989).

Ampicillin or amoxicillin in combination with gentamicin are the most effective drugs for the treatment of human listeriosis. Tetracycline, co-trimoxazole and erythromycin are suggested for therapy in case of allergy against ampicillin (Hof, 1991).

Encephalitis of ruminants has been the most frequently recognised form of listeric infection among nonhuman animals affecting sheep, goat and cattle. In the early stages, the animal tends to lean against stationary objects as if unable to stand unsupported and if the animal walks, it often moves in a circle thus the term ''circling disease'' (Gray and Killinger, 1966).

1.6 *LISTERIA* IN THE ENVIRONMENT

Weis and Seeliger (1975) showed that *L.monocytogenes* occurred in a high proportion of plants, soil and water samples and in faeces of some

animals. From their observations, they suggested that *L.monocytogenes* and other *Listeria* spp. exist as saprophytic organisms in the soil and on plants. Welshimer and Donket-Voet (1971) also suggested that the soil-plant environment was a reservoir for *L.monocytogenes* and survival was influenced by moisture content.

Silage has also been shown to be a reservoir for *L.monocytogenes*, particularly poor quality mouldy silage (Fenlon, 1985). Silage was implicated in a suspected outbreak of listeriosis in calves. Later it was shown that the silage contained *L.monocytogenes* at high levels (> 12,000 *L.monocytogenes*/g) (Fenlon 1986). In a flock of housed sheep, 19 ewes died after eating poor quality silage containing *L.monocytogenes* (Low and Renton, 1985).

L.monocytogenes appears to be a normal resident of the intestinal tract of animals as well as humans. *L.monocytogenes* can be shed in both the milk and faeces of apparently healthy cows (Fedio and Jackson, 1992). Watkins and Sleath (1981) showed that *L.monocytogenes* was present in sewage, sewage sludge and river water and that sewage sludge sprayed onto land showed no detectable reduction in numbers of *L.monocytogenes* during the 8 weeks after spraying.

These results suggest that there is a considerable reservoir of *L.monocytogenes* in the environment and in the human and animal population and thus allows *L.monocytogenes* easy access to food products during various stages of production, processing and distribution.

1.7 ISOLATION AND IDENTIFICATION OF *LISTERIA* SPECIES

Isolation of *L.monocytogenes* from food has in the past proved difficult and time consuming due to the presence of other competing microflora in the sample. In 1961, Seeliger recommended a cold enrichment technique in which the sample was incubated at 2-5°C for 4-12 weeks then inoculated into dextrose-tryptone broth and kept refrigerated for a further 4-6 weeks, subculturing onto Blood Agar (BA) and Tryptose Agar at weekly intervals to look for colony growth. At this time, this method could improve recovery of *L.monocytogenes* over direct plating due to the psychrotrophic nature of *L.monocytogenes* and the refrigeration temperatures reducing the competing microflora present. Due to the extended recovery time for *L.monocytogenes* other methods had to be adopted. These included the use of selective agar medium coupled with the oblique illumination of colonies (Henry, 1933), selective enrichment broths and alternative ''real time'' methods for isolation of *L.monocytogenes* from food. All these methods are outlined below.

Direct plating procedures using selective plating media do not reliably isolate *Listeria* from food. Prior enrichment of some kind is usually required. The main selective agars developed and adopted include Modified McBride's (Lovett, 1988), Lithium chloride phenylethanol-moxalactam (LCPMA) (Lee and McClain, 1986), Modified Vogel-Johnston (Buchannan *et al.*, 1988), PALCAM (Van Netten *et al.*, 1988) and Oxford Agar (Curtis *et al.*, 1989).

Agars such as PALCAM and Oxford have an advantage over other agars such as LCPMA as *Listeria* spp. can be visually differentiated from other non-

Listeria colonies due to colony morphology and colour without the need for Henry's illumination test (Henry, 1933).

There have been several evaluations of the different selective media and enrichment broths for isolation and enumeration of *Listeria* spp. in different food types (Bannerman and Bille, 1988; Dominguez *et al.*, 1988; Golden *et al.*, 1988; Westöö and Peterz, 1992; Poysky *et al.*, 1993). So far no single medium has emerged as superior for all food types.

Specific selective agents have now been added to enrichment media to shorten the incubation period required to isolate *Listeria* spp. from food. Nalidixic acid, acriflavine and cycloheximide have all been added to a Tryptone Soya Yeast Extract Broth (TSYEB) to form the basis of the commonly used Food and Drug Administration (FDA) *Listeria* Enrichment Broth (LEB) for detection of the organism in foods other than meats (Lovett, 1988). The incubation period in enrichment media has been reduced to 2 days and samples are then plated onto Oxford Agar and LCPMA and incubated at 30°C and 35°C respectively for 24-48 h (Hitchins and Tran, 1990). The current International Dairy Federation procedure for the detection of *Listeria monocytogenes* in milk and milk products uses LEB at 30°C, with plating onto Oxford Agar after 48 h.

Another method, the United States Department of Agriculture (USDA) method, was developed for isolation of *Listeria* spp. in meat and poultry (Lee and McClain, 1986; McClain and Lee, 1988). Since this study only involved dairy products, information on media for isolation of *Listeria* spp. from non-dairy products will not be included.

The identification of isolates of *Listeria* to the genus and species level is executed by a series of biochemical reactions, haemolytic activity and tumbling motility (see section 2.2.3). Differentiation between the *Listeria* spp. is outlined in Table 1.

The identification of *Listeria* spp. can also be carried out using commercial identification kits which give a quicker identification than by traditional methods. These include ROSCO System (Kerr *et al.*, 1991), MAST-ID (Kerr *et al.*, 1990), MICRO-ID (Robison and Cunningham, 1991; Bannerman *et al.*, 1992), API Listeria (Bille *et al.*, 1992) and API Coryne (Kerr *et al.*, 1993).

Alternative methods for isolation of *L.monocytogenes* from food without the traditional isolation and identification steps includes the Enzyme-Linked Immunosorbent Assay (Mattingly *et al.*, 1988; Vanderlinde and Grau, 1991 and Noah *et al*, 1991), Flow Cytometry (Donnelly and Baigent, 1986), a Polymerase Chain Reaction Assay (Bessesen *et al.*, 1990; Thomas *et al.*, 1991 and Fluit *et al.*, 1993), Immunomagnetic Separation (Skjerve *et al.*, 1990) and a Gene Probe assay (Notermans *et al.*, 1989 and Chewevert *et al.*, 1989).

1.8 LISTERIOSIS OUTBREAKS

The first confirmed report of listeric infection in man was in 1929 (Gray and Killinger, 1966), three years after it was first isolated (Murray *et al.*, 1926). It was not until 1957 that foodborne listeriosis was first suspected involving raw milk (Ryser and Marth, 1991). In 1981, *L.monocytogenes* was shown to be a foodborne pathogen when an outbreak of listeriosis in Canada was linked with consumption of coleslaw (Schlech *et al.*, 1983). 34 perinatal cases and 7 cases in adults were identified. The case fatality rate for infants

was 27%, 19 intrauterine deaths and 2 adult deaths occurred. The epidemic strain was subsequently isolated from an unopened packet of the coleslaw product. Subsequently cases of ovine listeriosis were found to have occurred in the flocks of a local farmer who had fertilised his cabbage fields with raw sheep manure.

The next outbreak occurred in Massachusetts in 1983 (Fleming *et al.*, 1985) and involved 49 cases of listeriosis. The case-fatality was 29% in both the adult and neonatal group. An epidemiologic investigation identified a specific brand of pasteurised milk as the vehicle of infection although *L.monocytogenes* was never isolated from the product. Cases of bovine listeriosis had occurred in the cows from which milk was supplied to the dairy. There was no evidence of improper pasteurisation at the plant and this gave rise to the first suggestion that *L.monocytogenes* had an increased heat resistance compared to other psychrotrophs.

A large outbreak of listeriosis occurred in California in 1985 (James *et al,*, 1985 and Linnan *et al.*, 1988). 142 cases of listeriosis occurred over an 8 month period resulting in 29 fetal or neonatal deaths and a case-fatality rate for 49 nonpregnant adults of 33%. A single producer of a Mexican-style cheese was identified as the source of the outbreak. The epidemic strain was isolated from unopened packs of the cheese in which unpasteurised milk had been incorporated into the finished product. This was highlighted due to the volume of milk being delivered to the plant on a number of occasions being greater than the plant pasteuriser could pasteurise in a single day.

Another outbreak due to consumption of soft cheese occurred in Switzerland during 1983-1987 (Bille, 1990). This involved 122 listeriosis cases with a case-fatality rate of 28%. Initially, a source of infection could not be found. Following a survey of dairy products for the presence of *Listeria* spp. in Switzerland, the source of the outbreak was traced to the surface of a Vacherin Mont d'Or soft cheese. In November 1987, all products were recalled from the market and the number of new cases dropped.

There was a near doubling in the incidence of human listeriosis in the UK between 1985 and mid 1989 followed by a sharp decline (McLauchlin *et al.*, 1991). Cardiff Public Health Laboratory examined a paté from the refrigerator of a patient suspected of having suffered food poisoning and isolated *L.monocytogenes.* Following a local survey of this food, 51% of samples were shown to be contaminated with *L.monocytogenes* (Cumber *et al.*, 1991). In July 1989, a Government health warning was issued advising vulnerable individuals to avoid eating paté (Anon, 1989). Gilbert *et al.* (1993) showed that the high rate of contamination detected in 1989 was due to a single paté manufacturer and levels of contamination with *L.monocytogenes* in 1990 were lower as a result of this single manufacturer no longer producing paté. McLauchlin *et al.* (1991) concluded that the contamination of paté was likely to have contributed to the increase in the incidence of listeriosis between 1987 and 1989. The number of cases and resulting deaths were unknown.

The latest outbreak of listeriosis occurred in France (Anon, 1993a and Salvat *et al.*, 1995) involving 279 cases between May and December in 1992,

which killed 63 people and caused 22 women to miscarry. It was not until the beginning of 1993 that the source of the outbreak was traced to jellied pork tongues. This was pin-pointed in a survey of the eating habits of 144 people involved in the outbreak. Researchers found that 46% of these people had eaten this product and it was later shown that *L.monocytogenes* was widespread in this type of product.

In all of these epidemic foodborne listeriosis outbreaks, isolates of *L.monocytogenes* have always been shown to be serotype 4b. There have also been a number of sporadic cases of listeriosis involving only one person in which the source of the outbreak was isolated from a particular food. *L.monocytogenes* was isolated from alfalfa tablets and soft cheese (Farber *et al.*, 1990), Anari whey cheese (Azadian *et al.*, 1988 and McLauchlin *et al.*, 1990), cooked chicken and vegetable rennet (Kerr *et al.*, 1988a). Other foods implicated in sporadic foodborne cases of listeriosis have included fish, turkey frankfurters, human breast milk, homemade sausage, salted mushrooms, cajan meat and rice sausage, raw milk, smoked cod roe, ice cream, fresh cream and pork sausage (Farber and Peterkins, 1991). The *L.monocytogenes* strains isolated were shown to be serotype 1/2a, 1/2b, 4 and 4b.

Between 1967 and 1982, fewer than 100 cases of listeriosis were reported annually in England, Wales and Northern Ireland. The number of cases started to rise slowly in the 1980s. By 1987, 259 cases were reported, with 291 in 1988 and 250 in 1989. By 1990, the number of cases had dropped back to those reported prior to the 1987 upsurge (McLauchlin *et al.*, 1991). A similar pattern of listeriosis reported cases have been shown in

Scotland, with the peak occurring between 1987 (36 cases), 1988 (40 cases) and 1989 (31 cases) followed by a sharp decline in listeriosis reported cases (Anon, 1993b).

It would seem that the warning given by the Department of Health advising pregnant women and immunocompromised people to avoid eating soft cheese, to adequatly reheat cook-chilled meals (Cumber *et al*, 1991), avoid paté (Anon, 1989) and the removal of a source of *L.monocytogenes* shown to be a particular brand of paté (Gilbert *et al.*, 1993) has all helped to greatly reduce the number of reported cases of listeriosis in the UK.

1.9 INCIDENCE OF *L.MONOCYTOGENES* IN FOOD

As previously mentioned, it was only in 1981 that the association with *L.monocytogenes* in foods as a source of infection was demonstrated. Since then, particular food types have been shown to be vulnerable to contamination by *L.monocytogenes*, in particular milk and dairy products. These products have been studied extensively due to the known association with foodborne listeriosis (Fleming *et al.*, 1985; James *et al.*, 1985; Linnan *et al.*, 1988; Bille, 1990; Farber *et al.*, 1990; Azadian *et al.*, 1988; McLauchlin *et al.*, 1990).

The presence of *L.monocytogenes* in milk has been shown in many studies around the world. Harvey and Gilmour (1992) isolated *L.monocytogenes* in 33.3% of the raw milk samples obtained at 4 milk processing centres in Northern Ireland. Dominguez-Rodriguez *et al.* (1985) reported an incidence of *L.monocytogenes* in dairy silos in Spain at 45.3% while Fernandez-Garayzabel

et al.(1987) isolated *L.monocytogenes* from 44.8% of samples of dairy silos in the same country two years later. Moura *et al.* (1993) isolated *L.monocytogenes* from 9.5% of raw milk samples from a Brazilian dairy plant.

The incidence of *L.monocytogenes* has been shown to be much lower in farm bulk tanks than in large industrial dairy silos as outlined previously. Fenlon and Wilson (1989) isolated *L.monocytogenes* in 4.8% of bulk milk tanks in North-East Scotland. This level was similar to results obtained from other studies of *L.monocytogenes* in farm bulk tank milks (Beckers *et al.*,1987; Lovett *et al.*, 1987; Fedio and Jackson, 1990; Harvey and Gilmour, 1992; Rea *et al.*, 1992; Rohrbach *et al.*, 1992).

There has been a variation in the seasonal incidence of *L.monocytogenes* reported in raw cows milk. Fenlon and Wilson (1989) reported a higher incidence of *L.monocytogenes* in the summer samples (3.8%) than in winter samples (1.0%). Rea *et al.* (1992) showed that there was a significant rise in the isolation rate for *Listeria* spp. (*L.monocytogenes* and *L.innocua*) between December and April from a base line of 0-5% during the spring and summer to 35-37% during the winter months while the cows were indoors. From this study it was shown that 9% of the silage being fed to the cows during the winter months contained *L.monocytogenes* and *L.innocua*.

L.monocytogenes has also been isolated in pasteurised cow milk samples at levels of 1.1% (Greenwood *et al.*, 1991), 1.05% (Harvey and Gilmour, 1992) and 0% (Moura *et al.*, 1993). Pasteurised milk has already been implicated in an outbreak of listeriosis in Massachusetts (see section 1.8). The

ability of *L.monocytogenes* to survive pasteurisation has been the subject of debate since this outbreak in 1983 (see section 1.10).

A national survey in England and Wales carried out between 1988-1989 for the occurrence of *Listeria* spp. in milk and dairy products showed that *L.monocytogenes* could be isolated from 8.2% of the samples tested. These included cows milk soft ripened and unripened cheese, cows milk hard cheese, goats milk cheese, ewes milk cheese, cows cream unpasteurised, yogurt and ice cream (Greenwood *et al.*, 1991). *L.monocytogenes* has also been isolated from soft ripened cheese, hard cheese, cream, yoghurt and ice-cream from other studies (Beckers *et al.*, 1987; Pini and Gilbert, 1988; Farber *et al.*, 1990; Farber and Peterkin, 1991).

L.monocytogenes has also been isolated from meat and meat products. It has now been associated with an outbreak of listeriosis in France involving jellied pork tongues (see section 1.8). It has been isolated from minced pork, minced beef, paté, ham, salami, pork sausages, hot dogs, and continental sausage (Schmidt *et al.*, 1988; Skovgaard and Morgan, 1988; Farber *et al.*, 1989; Morris and Ribeiro, 1989; Lacey, 1992; Salvat *et al.*, 1995).

Poultry and poultry products have also been shown to be contaminated with *L.monocytogenes* (Farber and Peterkins, 1991). More alarming has been the isolation of this organism from a range of cooked poultry products which had included ready-to-eat poultry, chilled poultry meals, and chicken salad (Kerr *et al.*, 1988a and 1988b; Gilbert *et al.*, 1989; Harvey and Gilmour, 1993).

L.monocytogenes has been isolated from a range of seafood. It has been

isolated from shrimp, crabmeat, lobster tails, fin fish, smoked fish, marinated fish and smoked salmon (Weagant *et al.*, 1988; Farber and Peterkin, 1991; Harvey and Gilmour, 1993).

L.monocytogenes has also been isolated from a range of vegetables (Heisick *et al.*, 1989). Cabbage has already been implicated in an outbreak of listeriosis (Schlech *et al.*, 1983). It was shown that cases of ovine listeriosis were found to have occurred in a flock of sheep of a local farmer who had fertilised his cabbage fields with raw manure. These cabbages were then used to manufacture the coleslaw implicated in the outbreak. Contamination of vegetables will occur in the soil as Welshimer and Donket-Voet (1971) have shown the soil-plant environment to be a reservoir for *L.monocytogenes.* It can also be shed in the faeces of cows (Fedio and Jackson, 1992) and this material is then used to fertilise the soil thus reintroducing *L.monocytogenes* into the soil. *L.monocytogenes* has also been shown to be present in prepacked salads ready for consumption (Sizmur and Walker, 1988).

1.10 THERMAL RESISTANCE OF *L.MONOCYTOGENES*

Pasteurised milk was implicated in an outbreak of listeriosis in Massachusetts in 1983 (Fleming *et al.*, 1985). No evidence of faulty pasteurisation was shown at the implicated dairy but the suggestion that *L.monocytogenes* was unusually thermotolerant was raised at this time.

Bradshaw *et al.* (1985) concluded that *L.monocytogenes* could not survive HTST pasteurisation (71.7^{o}C for 15 s) using the ''sealed glass tube'' method in raw milk. Others have also reported that *L.monocytogenes* could not survive

HTST protocols using different methods (Donnelly *et al.*, 1987, Beckers *et al.*, 1987; Mackay and Bratchell, 1989; Lovett *et al.*, 1990).

Doyle *et al.* (1987) reported that the intracellular location of the organism did increase the thermal resistance of *L.monocytogenes* when the organism was contained within polymorphonuclear leukocytes. Bunning *et al.* (1988) showed that when *L.monocytogenes* was located within bovine phagocytes, this did not increase the thermal resistance of the organism to heating. Farber *et al.* (1988) reported that *L.monocytogenes* was not recovered from milk heated at 69°C and above for 16 s regardless of the extracellular and intracellular location of *L.monocytogenes.*

Exposure of *L.monocytogenes* to heat treatments of 43-48°C for up to 30 minutes has been shown to induce increased resistance to further heating at higher temperatures such as 62.8°C when compared to control cultures (Knabel *et al.*, 1990). This is called the heat shock response. Most microorganisms will respond to sublethal heat treatment by synthesizing a set of proteins called the heat shock proteins (Craig, 1985). The heat shock response has been demonstrated in milk (Fedio and Jackson, 1989) and in meat (Farber and Brown, 1990).

Acid shock treatment prior to heating has been shown to increase the heat resistance of *L.monocytogenes* in whole milk. It appears that acidification with hydrochloric acid (but not acetic acid) prior to final heating at 58°C can enhance the heat resistance of this organism (Farber and Pagotto, 1992). This study also showed that a gradual or precipitous drop in pH due to the acidification with hydrochloric acid may help to enhance the survival of

L.monocytogenes. It has been suggested that these conditions could be simulated in foods by the introduction of starter cultures such as would be used in cheese manufacture.

Heat-injured *Listeria* spp. were examined for their ability to repair in pasteurised milk. It was shown that repair of *L.innocua* and *L.monocytogenes* at 4, 10, 26 and 37°C took between 8-19 days, 4 days, 13 hours and 9 hours respectively (Meyer and Donnelly, 1992). It was also shown that current *Listeria* detection techniques were not adequate for the detection of injured *Listeria* spp. This implies that dairy products given a sub-lethal heat treatment could recover no *L.monocytogenes* on testing but during ripening of the product at between 2-8°C, heat-injured *L.monocytogenes* could repair during the prolonged ripening period given to dairy products such as cheese and result in an unsafe product.

Smith and Archer (1988) also showed that current selective media for isolation of *L.monocytogenes* were not satisfactory for the recovery of heat-injured cells. A new *Listeria* enrichment broth was then developed to help repair and resuscitate heat-injured *L.monocytogenes* and *L.innocua* (Busch and Donnelly, 1992). When the *Listeria* spp. were incubated in this broth, the heat-injured *Listeria* spp. were found to be completely repaired within 5 hours. It was after this initial recovery period that the supplements were then added to make it a selective *Listeria* enrichment broth.

TABLE 1. Differential characters of *Listeria* spp.

Species	Haemolysis*	CAMP test		Acid from				Reduction of nitrate to nitrite
		S.aureus	*R.equi*	D-mannitol	L-rhamnose	D-xylose	α-methyl D-mannoside	
L.monocytogenes	+	+	-	-	+	-	+	-
L.innocua	-	-	-	-	V	-	+	-
L.ivanovii	++	-	+	-	-	+	-	-
L.seeligeri	W	+	-	-	-	+	V	-
L.welshimeri	-	-	-	-	V	+	+	-
L.grayi	-	-	-	+	V	-	+	V

*+ = moderate; ++ = strong; W = weak + = positive - = negative V = varaible reaction

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 CULTURES

The *Listeria* organisms and strains used in this study were all dairy isolates and were *L.monocytogenes* (St Ivel Technical Centre, Wootton Bassett, Swindon, (SITC) 12/1A), *L.monocytogenes* (SITC 404/2), *L.monocytogenes* (National Collection of Type Cultures (NCTC) 7973), *L.monocytogenes* Scott A (Milk Marketing Board, Thames Ditton, Surrey, (MMB) 7), *L.monocytogenes* (MMB 8), *L.innocua* (SITC 112/2A/6), *L.innocua* (SITC 236/2/8), *L.innocua* (NCTC 11288), *L.murrayi* (NCTC 10812) and *L.murrayi* (MMB 4).

L.monocytogenes and *L.innocua* were isolated from raw cows' milk analysed during this study (see Chapter 3). Some of these isolates were used in subsequent work (see chapter 8). *L.monocytogenes* isolates used were L519, L527, L533, L560 and L580 while *L.innocua* isolates used were L510, L523, L534, L553 and L585. All of these isolates are now contained in the microbiology culture collection of the Hannah Research Institute, Ayr, Scotland.

The fungal cultures and strains used in this study were *Penicillium roqueforti* (*P.roqueforti*) (International Mycology Institute (IMI) 148775), *P.roqueforti* (IMI 24313), *P.roqueforti* (IMI 129207), *P.roqueforti* (PRB2) and (PRB6) commercial strains from Lacto. Labo (Group Rhône-Poulenc), *P.roqueforti* (NO-N) commercial strain from Centro Sperimentale Del Latte, *P.roqueforti* (Bleu 18) commercial strain from Texel, Saint-Romain, France, *P.camemberti* (IMI 129208), *P.camemberti* (IMI 27831), *P.camemberti* (IMI

214010), *P.camemberti* (Niege) and (Sam 2) commercial strains from Lacto. Lab (Group Rhône-Poulenc) and *P.candidum* (S11) commercial strains from Texel, Saint-Romain, France.

The remaining bacterial cultures used in this study were *Pseudomonas fluorescens* (National Collection of Dairy Organisms (NCDO 2085) now changed to National Collection of Food Bacteria (NCFB), *Citrobacter freudii* (Hannah Research Institute collection (GTE 022), *Acinetobacter lwoffi* (GTE 024), *Klebsiella ozoaenae* (GTE 019), *Staphylococcus aureus* (NCTC 1803), *Rhodococcus equi* (NCTC 1621) and *Lactococcus lactis* (E9275), (SL391) and (SL213) commercial strains from Marschall-Eurozyme U.K. (Rhône-Poulenc).

The cheese starter cultures used were MA 400 EZAL 1 and MA 011. MA 400 EZAL 1 is a commercial mixed-strain culture consisting of *Lactococcus lactis subsp lactis* (70%), *Lactococcus lactis subsp diacetylactis* (20%) and *Lactococcus lactis subsp cremoris* (10%). Both are specifically prepared for direct vat inoculation used for cheese manufacture and were supplied from Fullwood and Bland Ltd. Both were used following the manufacturers instructions.

2.1.2 MAINTENANCE OF CULTURES

A single bacterial colony was selected for each bacterial isolate and inoculated onto a Tryptone Soya Yeast Extract Agar (TSYEA) slope (see section 2.1.4) and incubated at 37°C for 24-48 h, or until growth had occurred. All fungal cultures were inoculated onto Czepak Agar (see section 2.1.4) and incubated at 21°C for 5-7 days, or until growth had occurred. All these slopes were stored at 4°C. All the commercial fungal cultures were in a ready-to-use

form as a spore suspension or dry powder presentation.

All the bacterial cultures were sub-cultured at least twice onto TSYEA before being used in experiments. All fungal cultures were sub-cultured onto Malt Extract Agar (MEA) (see section 2.1.4) before being used in experiments.

2.1.3 MEDIA

All culture media used in this study was purchased from Oxoid (Unipath, Basingstoke, U.K.) and were prepared according to the manufacturers instructions. All media, diluents and broths were prepared with distilled water. All media were autoclaved at 121°C for 15 min, cooled to 45°C then supplements added aseptically as required and poured into petri dishes using an automatic plate pourer (Cherwell Laboratories). All plates were stored inverted at 2-8°C and used within 7 days. All diluents and broths were autoclaved at 121°C for 15 min and cooled before use.

2.1.4 MEDIA COMPOSITION

Blood Agar (BA)

Formula	g/litre
Proteose peptone	15.0
Liver digest	2.5
Sodium chloride	5.0
Agar	12.0

The agar was cooled to 45°C, 70ml sterile Sheep blood added and mixed well. It was then dispensed into sterile petri dishes.

CAMP Agar

Base medium

Formula	g/litre
Proteose peptone	15.0
Liver digest	2.5
Yeast extract	5.0
Sodium chloride	5.0
Agar	12.0

Top medium

Formula	g/litre
Proteose peptone	15.0
Liver digest	2.5
Yeast extract	5.0
Sodium chloride	5.0
Agar	12.0

The agar was cooled to 45°C, 70ml sterile Sheep blood added and mixed well.

Complete CAMP medium

The base medium was distributed in sterile petri dishes in quantities of 10ml and allowed to solidify. A very thin layer of top medium was poured over the base using no greater than 3ml per plate. This was allowed to solidify in an even layer.

Carbohydrate fermentation broth

Base medium

Formula	g/litre
Proteose peptone	10.0
Beef extract	1.0
Sodium chloride	5.0
Bromocresol purple	0.02

It was dispensed in 9ml volumes and autoclave as before.

Carbohydrate supplements:	g/100ml
D-mannitol	5.0
L-rhamnose	5.0
D-xylose	5.0
α-methyl D-mannoside	5.0

It was sterilized by filtration through a 0.45μm filter (Sartorius).

Complete Carbohydrate fermentation broth

Formula	
Base medium	9.0ml
Carbohydrate supplement	1.0ml

Czapek Agar

Formula	g/litre
Sodium nitrate	2.0
Potassium chloride	0.5

Magnesium glycerophosphate	0.5
Ferrous sulphate	0.01
Potassium sulphate	0.35
Sucrose	30.0
Agar	12.0

Listeria Enrichment Broth (LEB)

Formula	g/litre
Tryptone soya broth	30.0
Yeast extract	6.0

Supplement: each vial sufficient for 500ml of medium

Nalidixic acid	20.0mg
Cycloheximide	25.0mg
Acriflavine hydrochloride	7.5mg

Each supplement was reconstituted with 2ml of sterile water. The medium was cooled to 50°C and the contents of the required number of vial supplements aseptically added. The complete LEB was mixed well and distributed into sterile containers in 225ml volumes.

Listeria Repair Broth (LRB, Busch and Donnelly, 1992)

Formula	g/litre
Tryptone soya broth	30.0
Yeast extract	6.0
Glucose	5.0
3-N-morpholinepropanesulfonic acid (MOPS)	
(Free acid)	8.5

MOPS (Sodium salt)	13.7
Ferrous sulphate	0.3
Magnesium sulphate	4.94
Pyruvate	10.0

Ferrous sulphate was added to distilled water and dissolved. The TSB, Glucose, Yeast extract and MOPS were then added , heated and dissolved completely. Magnesium sulphate was then added and boiled. It was removed from the heat and Pyruvate added when cool. It was then well mixed. The solution was clear and deep gold to slightly reddish in colour. It was dispensed into sterile containers in 225ml volumes and autoclaved as before. Instruction for manufacture were not contained in the Busch and Donnelly (1992) publication but were obtained by personal communication from their laboratory.

Supplements: same as for LEB

The supplements for LRB were added 5h after the start of incubation to assist the revival of stressed bacteria.

Malt Extract Agar (MEA)

Formula	g/litre
Malt extract	30.0
Mycological peptone	5.0
Agar	15.0

Maximum Recovery Diluent (MRD)

Formula	g/litre
Peptone	1.0
Sodium chloride	8.5

Milk Agar (MA)

Formula	g/litre
Yeast extract	3.0
Peptone	5.0
Milk solids	1.0
Agar	15.0

Nutrient Broth No.2 (NA)

Formula	per litre
'Lab-Lemco' powder	10.0g
Peptone	10.0g
Sodium chloride	5.0g

Nutrient Broth No.2 with 15% Glycerol

Formula	per litre
'Lab-Lemco' powder	10.0g
Peptone	10.0g
Sodium chloride	5.0g
Glycerol (15% v/v)	150ml
Distilled water	850ml

Oxford Agar

Formula	g/litre
Columbia blood agar base	39.0
Aesculin	1.0

Ferric ammonium citrate	0.5
Lithium chloride	15.0

Supplement: each vial sufficient for 500ml of medium

Cycloheximide	200mg
Colistin sulphate	10mg
Acriflavine	2.5mg
Cefotetan	1.0mg
Fosfomycin	5.0mg

Each vial was reconstituted with 5ml of ethanol/sterile distilled water (1:1). The medium was cooled to 50°C and the contents of the required number of vial supplements aseptically added. It was well mixed and poured into sterile petri dishes.

Tryptone Soya Broth (TSB)

Formula	g/litre
Pancreatic digest of casein	17.0
Papaic digest of soybean meal	3.0
Sodium chloride	5.0
Dibasic potassium phosphate	2.5
Glucose	2.5

Tryptone Soya Yeast Extract Agar (TSYEA)

Formula	g/litre
Tryptone	15.0
Soya peptone	5.0

Sodium chloride	5.0
Agar	15.0
Yeast extract	6.0

Tryptone Soya Yeast Extract Broth (TSYEB)

Formula	g/litre
Tryptone soya broth	30
Yeast extract	6.0

2.2 METHODS

2.2.1 SPIRAL PLATE COUNT METHOD

The Spiral Plater Model D (Spiral Systems, Inc) was used following the instructions in the user manual. The sample was taken up into the stylus by means of a vacuum pump and stored in a previously disinfected syringe. A pre-poured agar plate was placed onto the turntable and the stylus lowered onto it. When the system was switched on, the turntable rotated and the sample (approximately 0.05ml) was distributed onto the surface of the agar plate in an Archimedes spiral. The stylus then returned to the start position and the syringe was disinfected and washed with sterile water ready for use again.

The amount of sample on the plate decreased as the spiral moved out towards the edge of the plate. The quantity of sample deposited on any given area of the plate was determined by following the manufacturers instructions. After inoculation, the plates were incubated as required. Following incubation, the total number of Bolonies on the plate were counted using a Plate counter (Spiral Systems, Inc) and counting grid to determine colony forming units/ml

(cfu/ml). Each counting grid corresponded with a known volume of sample deposited in that area so by counting the total number of colonies in that area and dividing by the sample volume, the cfu/ml could be calculated.

2.2.2 BACTOMETER IMPEDANCE DETECTION METHOD

A Bactometer M64 was used in this study (BioMérieux UK Ltd, Basingstoke, Hants). Sterile disposable modules each with 16 wells were used throughout. Each well had electrodes at the base of the well. Each well had a maximum capacity of 2ml but was only ever filled with 1ml of broth. All the modules were incubated at $30^{o}C$ for 50 h or until a detection time (dt) in hours was given . A dt was given once a defined level of $1x10^{6}$ cfu/ml had been reached inside the well. The change in impedance measured as capacitance of the medium was monitored using the Bactometer M64. Changes in capacitance are associated with changes taking place in the medium at the electrodes in the wells. As microorganisms metabolise nutrients they create new end products in the medium. Generally, uncharged or weakly charged substrates are transformed into highly charged end products. Changes in the current measured as capacitance can be directly related to growth of the microorganisms in the well (Eden and Eden, 1984).

2.2.3 IDENTIFICATION OF *LISTERIA* SPECIES

In this study, all *Listeria* spp. were isolated and identified using the International Dairy Federation standard for milk and milk products (detection of *L.monocytogenes*) (Anon, 1990). This method was selected as it was a

recognised standard method for the products examined throughout this study. This method included preparation of the test sample, incubation, isolation and presumptive identification and confirmation of isolates. Tests carried out on a presumptive *Listeria* colony were catalase (see section 2.2.6), oxidase (see section 2.2.7), tumbling motility (see section 2.2.9), haemolysis (see section 2.2.5), carbohydrate fermentation (see 2.2.8) and the CAMP test (see section 2.2.4) which was carried out only when a weak or doutful β-haemolytic reaction was given on BA. Later on in the study, API Listeria biochemical identification kits (BioMérieux UK Ltd) were also used to confirm any presumptive *Listeria* colonies (Bille *et al.*, 1992). Differentiation between the *Listeria* spp. using these tests are outlined in Table 1.

2.2.4 CAMP TEST

The Christie-Atkins-Munch-Peterson (CAMP) test (Christie *et al.*, 1944) was performed by streaking *S.aureus* and *R.equi* (see section 2.1.1) in single lines across a CAMP blood agar plate (see section 2.1.4) so that the two cultures are parallel and diametrically opposite. The test strain was streaked at right angles to these cultures so that the test culture and reaction culture did not touch. Several test strains could be streaked onto the same plate. Control cultures of *L.monocytogenes*, *L.innocua* and *L.ivanovii* were also simultaneously streaked across. The plates were then incubated at 37°C for 18-24 h. Positive reactions were indicated by an enhanced zone of β-haemolysis at the intersection of the test strain with either the *S.aureus* and *R.equi* culture. *L.monocytogenes* and *L.seeligeri* show a positive CAMP reaction with *S.aureus*

but not with *R.equi*. *L.ivanovii* reacts with *R.equi* but not with *S.aureus*. The other *Listeria* spp. show negative CAMP reactions with both reaction cultures.

2.2.5 HAEMOLYSIS

Using a surface dry BA plate (see section 2.1.4), a grid was drawn on the plate bottom marking 20-25 spaces/plate. A typical colony was selected from a TSYEA plate (2.1.4) and stabbed into one space using an inoculating needle. Positive and negative controls were simultaneously inoculated. After 48 h incubation at 37°C, the stabs were examined. *L.monocytogenes* shows narrow, slight zones of clearing (β-haemolysis), *L.innocua* should show no clear zone around the stab while *L.ivanovii* usually shows wide, clearly delineated zones of β-haemolysis.

2.2.6 CATALASE

The catalase test was performed by selecting a typical colony from a TSYEA plate and suspending it in a drop of 3% hydrogen peroxide solution on a clean glass slide. A positive reaction is demonstrated by the formation of gas bubbles. All *Listeria* spp. give a catalase positive reaction.

2.2.7 OXIDASE

The oxidase reagent used was a Kovacs' 1% tetramethyl-p-phenylenediamine dihydrochloride with 0.1% ascorbic acid (MacFaddin, 1990). It must be stored at 4°C in a dark bottle. The solution is

colourless and should not be used if it turns deep blue. The oxidase test was performed by soaking a filter paper with the oxidase reagent and selecting a typical colony from a TSYEA plate (see section 2.1.4) and streaking it across the paper. Development of a purple-blue colour within 1 minute indicates a positive reaction. All *Listeria* spp. give an oxidase negative reaction.

2.2.8 CARBOHYDRATE FERMENTATION

Each carbohydrate fermentation broth (see section 2.1.4) was inoculated with one loopful of a TSYEB culture previously incubated at 37°C for 24 h. The broths were then incubated at 37°C for 7 days. A positive reaction is indicated by a yellow colour due to acid formation. Fermentation reactions by the different *Listeria* spp are outlined in Table 1.

2.2.9 TUMBLING MOTILITY

A typical colony was selected from a TSYEA plate and inoculated into TSYEB (see section 2.1.4) and incubated at 25°C for 6-24 h. After incubation, a hanging drop suspension of the broth was prepared by placing a drop of the broth on a cover slide and mounting it onto a glass slide with a ring of vaseline seperating both. The hanging drop was examined using an oil immersion phase contrast objective. *Listeria* spp. appear as slim, short rods with slight rotating or tumbling motility.

2.2.10 HENRY ILLUMINATION

Colonies were streaked onto the surface of TSYEA (see section 2.1.4) in

a manner which allowed well isolated colonies to develop. Plates were incubated at 37°C for 24 h or until growth was satisfactory. The plates were examined using a white light powerful enough to illuminate the plates and striking the bottom of the plate at a 45 degree angle. When examined in this obliquely transmitted light (Henry illumination) from directly above the plate, colonies of *Listeria* spp. exhibit a blue colour and a granular surface.

2.2.11 STATISTICAL ANALYSIS

All statistical analysis was carried out using one-way Analysis of Variance, Minitab (Minitab Release 7, Minitab Inc, PA).

CHAPTER 3

INCIDENCE OF *LISTERIA* SPECIES IN MILK AND CHEESE

3.1 INTRODUCTION

Listeria monocytogenes has been implicated as the causative agent in several outbreaks of foodborne listeriosis involving milk (Fleming *et al.*, 1985) and milk products, particularly several types of soft cheese (James *et al.*, 1985; Linnan *et al.*, 1988; Azadian *et al.*, 1989; Bille, 1990).

Beckers *et al.* (1987) isolated *L.monocytogenes* in 10 out of 69 samples of imported cheese (*ie.* Brie and Camembert), all made from raw milk. The raw milk supply was indicated as a possible source of contamination of these products. *L.monocytogenes* is widespread in the farm environment and contaminated silage is thought to be the main source of infection for listeriosis in animals (Fedio and Jackson, 1992).

There have been many studies on the incidence of *L.monocytogenes* in cow milk (Dominguez-Rodriguez *et al.*, 1985; Lovett *et al.*, 1987; Beckers *et al.*, 1987; Fenlon and Wilson, 1989; Rohrbach *et al.*, 1992; Rea *et al.*, 1992), but so far no studies have been published on the incidence of *L.monocytogenes* in raw sheep and goat milk. The seasonal and overall incidence of *L.monocytogenes* has varied between these studies and inevitably between countries.

The overall incidence of *Listeria* spp. (in particular *L.monocytogenes*) in milk and dairy products were studied to determine the levels of these organisms in Central Scotland and to highlight if any or all of these products could be a potential source of a listeriosis outbreak. All of these products were sampled

over a one year period to highlight any seasonal incidence of *L.monocytogenes* and *Listeria* spp. perhaps as a result of changing feeding patterns (from grass to silage), environment (from pasture land to indoor housing during Autumn and Winter months) and also changes due to the lactation cycle of these animals.

This work investigated the seasonal and overall incidence of *Listeria* spp. in raw sheep, cow and goat milks, pasteurised cow milk, raw and pasteurised cheese produced using different milk types. Total counts of milk and cheese samples were taken to show if there was any correlation between the total counts and incidence of *Listeria* spp., particularly *L.monocytogenes.*

3.2 EXPERIMENTAL PROCEDURES

3.2.1 SAMPLES

Over a one year period commencing on March 1991, 60 pasteurised cow milk samples were collected monthly. Over a further one year period commencing on June 1992, a total of 21 raw sheep milk, 54 raw goat milk , 90 raw cow milk, 298 soft and semi-soft cheese and 7 hard cheese samples were obtained at regular monthly intervals from a number of dairies and farms in Scotland. Raw sheep and goat milk samples were obtained from collection tanks at three and ten farms, respectively. Raw cow milk samples were obtained from dairy silos at dairies. The cheese samples were obtained from four retail supermarkets (coded A-D) and from two farm cheese manufactures (coded S-1 and S-3). Due to lactation cycle of sheep and goats, samples were not available during November through to February for goat milk and during

November through to March for sheep milk samples. All samples were maintained at between 2-8°C during transportation to the laboratory and were processed that day.

3.2.2 ISOLATION OF *LISTERIA* SPP.

25 ml of each milk sample was aseptically added to 225 ml of LEB (see section 2.1.4) and incubated at 30°C for 48 h. 25 g of each cheese sample was aseptically added to 225 ml of LEB (see section 2.1.4) and incubated at 30°C for 48 h.

Total counts for *Listeria* spp. were obtained for these milk and blended cheese samples by spread plating (Stainer *et al.*, 1987) 2x500 µl samples onto duplicate Oxford agar plates (see section 2.1.4) and incubating at 37°C for 48 h. After incubation of the enrichment broths, 2x500 µl samples (with appropriate dilutions in MRD) (see section 2.1.4) were spread plated onto duplicate Oxford agar plates and incubated at 37°C for 48 h. Oxford agar plates were then examined for the presence or absence of typical *Listeria* colonies, *ie* all colonies which hydrolysed aesculin in the agar resulting in a blackening of the agar around the colony. Five presumptive *Listeria* colonies per positive sample were selected and their identity confirmed (Anon, 1990) (see section 2.2.3). Five confirmed *Listeria* colonies per sample were streaked onto TSYEA (see section 2.1.4) and after incubation were harvested using NB supplemented with 15% glycerol (see section 2.1.4) and this inoculum was used to coat glass beads inside a glass vial. These vials were then stored at -80°C.

3.2.3 TOTAL BACTERIAL COUNTS IN MILK AND CHEESE

Total bacterial counts were obtained for milk samples (except pasteurised milk) and blended cheese samples (with appropriate dilutions in MRD) on MA plates (see section 2.1.4) using a Spiral Plater Model D (Spiral Systems Inc) (see section 2.2.1) and incubated at 30°C for 72 h. All plates were counted using a Plate Counter (Spiral Systems Inc) (see section 2.2.1) to calculate cfu/ml and cfu/g respectively.

3.3 RESULTS

3.3.1 INCIDENCE OF *LISTERIA* SPECIES IN MILK AND CHEESE

The overall incidence of *Listeria* spp. isolated from raw cow, sheep and goat milk and pasteurised cow milk was 32.2%, 23.8%, 5.6% and 0% (Table 2). The *Listeria* spp. isolated from raw cow milk samples were *L.monocytogenes* (8.9%), *L.innocua* (21.1%) and *L.ivanovii* (2.2%). *L.monocytogenes* and *L.innocua* were isolated from raw sheep milk at 4.8% and 19% and from goat milk at 1.9% and 3.7%, respectively. No *Listeria* spp. were isolated from pasteurised cow milk.

L.monocytogenes was not detected from any of the milk samples examined by direct plating on Oxford agar but was isolated in 10 milk samples after enrichment procedures (Table 3). On four occassions *L.innocua* was isolated by direct plating on Oxford agar at levels of 1 cfu/ml from sheep milk during June and July and cow milk during August and November (Table 3). *L.innocua* was isolated in a further 21 samples after enrichment procedures. *L.ivanovii* was only isolated after enrichment procedures and only in cow milk samples.

The overall incidence of *Listeria* spp. isolated from cheese samples was 4.9%, with *L.monocytogenes* and *L.innocua* isolated at 1% and 3.9% respectively (Table 2). *L.innocua* was isolated by direct plating on Oxford agar from 6 cheese samples during July, September and November at levels of between $1x10^1$ to $1x10^3$ cfu/g (Table 4). *L.monocytogenes* was isolated by direct plating on Oxford agar from a single type of unpasteurised sheep milk cheese on three occasions during January, February and March at levels of between $1x10^1$ to $2.56x10^3$ cfu/g (Table 4). *L.innocua* was isolated in July 1993 from Half-fat blue cheese (supermarket code C) which was no longer on sale for the remainder of the survey.

The seasonal incidence of *L.monocytogenes* and other *Listeria* species in raw sheep, cow and goat milks during June 1992 to May 1993 are shown in Figures 1-3. The incidence of *Listeria* spp. in raw cows milk (Figure 1) was shown to be highest during the months of October and December while *L.monocytogenes* was at its lowest during this period. The results showed that the incidence of *L.monocytogenes* persisted during July through to September.

The incidence of *Listeria* spp. in raw sheep milk (Figure 2) were shown to occur during Summer and Autumn while *L.monocytogenes* was isolated during late spring. No seasonal incidence of *L.monocytogenes* and *L.innocua* in raw goats milk (Figure 2) could be shown.

The seasonal incidence of *L.monocytogenes* and *L.innocua* in cheese during June 1992 to May 1993 are shown in Figure 3. The results show *L.monocytogenes* was only isolated during January through to March while

L.innocua was isolated at its highest levels in July, September and November.

3.3.3 TOTAL BACTERIAL COUNTS IN MILK AND CHEESE

The monthly total bacterial counts in raw sheep, goat and cow dairy silo milks between June 1992 to May 1993 are outlined in Table 5. These counts were compared with the isolation of *Listeria* spp. (Table 3) using Analysis of Variance (see section 2.2.11). The results showed that there was no correlation between the total bacterial count and isolation of *Listeria* spp. from the milk samples.

The monthly total bacterial counts in cheese between June 1992 to May 1993 are shown in Table 6. Using Analysis of Variance (see section 2.2.11), it was shown that there was no correlation between the total bacterial count and isolation of *Listeria* spp. from the cheese samples (Table 4). Since the cheese used were fermented products, specific bacteria such as *Enterobacteriaceae* should have been counted.

3.4 DISCUSSION

The overall incidence of *Listeria* spp. in raw cow milk obtained from dairy silos at 5 processing centres was found to be 32.3%, with *L.monocytogenes*, *L.innocua* and *L.ivanovii* at 8.9%, 21.1% and 2.2% respectively. Harvey and Gilmour (1992) isolated *Listeria* spp. from 54% of milk samples obtained at 4 milk processing centres in Northern Ireland over a one year period, with *L.monocytogenes*, *L.innocua* and *L.seeligeri* isolated at 33.3%, 19% and 7.9% respectively. Dominguez-Rodriguez *et al.* (1985) reported an overall incidence of *Listeria* spp. in dairy silos in Spain at greater

than 85% with *L.monocytogenes* isolated at 45.3%. Fernandez-Garayzabal *et al.* (1987) isolated *L.monocytogenes* from 44.8% of samples of dairy silos in Spain. All these authors have reported a higher incidence of *L.monocytogenes* than was reported in this study. This could reflect the high quality of hygiene achieved by Scottish farms and dairies compared with other countries.

Harvey and Gilmour (1992) also isolated *Listeria* spp. at 8.8% in bulk tank milk in Northern Ireland with *L.monocytogenes* at 5.3% from 8 dairy farms. This level was similar to results obtained from other studies on incidence of *Listeria* in farm bulk tank milks (Beckers *et al.*, 1987; Lovett *et al.*, 1987; Fenlon and Wilson, 1989; Rea *et al.*, 1992). The incidence of *Listeria* spp. isolated by others and in this study from dairy silos was therefore far greater than reported levels in farm bulk tanks. Resampling the same small number of milk silos per month (*ie.* 5 dairy silos) may have increased the incidence of *Listeria* spp. isolated. This higher incidence is probably due to the mixing of a large number of farm milks in the silos, only one of which needs to contain a detectable level of *L.monocytogenes.* No single dairy silo gave continuous isolation of *Listeria* spp.

Sampling each month for one complete year enabled this study to highlight any seasonal incidence of *Listeria* spp. and particularly *L.monocytogenes* in dairy silo milk. These milk samples reflect the actual numbers of *L.monocytogenes* present in milk immediately before processing, unlike the examination of individual bulk tank milk samples which do not take

into account the effect of storage and bulking of the milk at the dairy processing centres.

The overall incidence of *L.innocua* was always greater than the incidence of *L.monocytogenes* in all the milk types sampled. On no occasion was *L.innocua* and *L.monocytogenes* isolated together from a single sample.

In this survey, *L.monocytogenes* and *L.innocua* were isolated from raw sheep milk at 4.8% and 19% and from goat milk at 1.9% and 3.7%, respectively. These results indicate that cheese manufactured using raw sheep or goat milk could possibly contain *L.monocytogenes.* The growth kinetics of *L.monocytogenes* have been examined in many cheeses (Ryser and Marth, 1987a; Ryser and Marth, 1987b; Papageorgiou and Marth, 1989: Dominguez *et al.*, 1987) and the organism was found to grow to high numbers in mould ripened soft-cheeses such as Camembert.

The overall incidence of *Listeria* spp. isolated from cheese samples was 4.9%, with *L.monocytogenes* and *L.innocua* isolated at 1% and 3.9% respectively (Table 2). The only type of cheese from which *L.monocytogenes* was isolated was a mould ripened semi-soft farm cheese manufactured from unpasteurised sheep milk. Levels of $1x10^{1}$-$2.56x10^{3}$ cfu/g were isolated by direct plating of the cheese onto Oxford agar. *L.innocua* was also isolated from this cheese by direct plating onto Oxford agar at levels of $2.8x10^{2}$-$7.75x10^{2}$ cfu/g. The raw milk used to produce this cheese was also sampled in the survey (S-3), but only *L.innocua* was isolated from the milk supply. This suggests that either the isolation procedures did not detect low levels of *L.monocytogenes* which subsequently multiplied in the cheese or that

L.monocytogenes was a post-manufacturing contaminant of the cheese, derived from an environmental source. It should also be noted however that milk from this source was not available for sampling between November 1992 to March 1993. The date of collection for milk used for cheese found to be contaminated with *L.monocytogenes* (sampled January to March) was also unknown.

The growth of *L.monocytogenes* in semi-soft cheese can be a potential hazard to certain members of the community if ingested (i.e. pregnant woman, infants, the elderly and immunocompromised people) (Gray and Killinger, 1966 and Seeliger, 1972). At present the infectious dose of *L.monocytogenes* in food is unknown.

It was apparent that a significant percentage of raw milks, in particular cows milk (8%), were contaminated with *L.monocytogenes.* It was not isolated in pasteurised cow milk in this survey of 60 samples so it can be concluded that pasteurisation is an efficient method for removal of *L.monocytogenes* from raw milk.

Listeria monocytogenes was not detected from any of the raw or pasteurised milk samples examined by direct plating on Oxford agar at a detection level of greater than 1 cfu/ml. The International Dairy Federation (Anon, 1990) recommends the use of Oxford agar as the isolation medium for isolation of *L.monocytogenes.* Harvey and Gilmour (1992) have also shown Oxford agar to be superior to Modified McBrides agar for isolation of *Listeria* spp. for milk samples.

It was shown that the highest levels of *Listeria* spp., particularly *L.innocua* occurred in raw cow milk during late Autumn and Winter while the highest levels of *L.monocytogenes* occurred during Summer and early Autumn. The seasonal incidence of *L.monocytogenes* and *Listeria* spp. in raw sheep milk

showed that the highest levels of *Listeria* spp. occurred during Summer and Autumn while the only incidence of *L.monocytogenes* to occur was during Spring. No seasonal incidence was found for *Listeria* spp. and *L.monocytogenes* in goats milk. The seasonal incidence of *L.monocytogenes* and *L.innocua* in cheese showed that *L.innocua* was isolated at its highest levels during months outwith the isolation of *L.monocytogenes.* These findings suggest that *L.monocytogenes* was only isolated when the levels of *Listeria* spp., particularly *L.innocua* were low.

The results of this survey show that raw sheep, goat and cow milk all contain *L.monocytogenes.* This highlights the potential of raw milks to be a source of listeric infection when used to manufacture a product such as raw milk soft-cheese which can support the continuous growth of *L.monocytogenes* (Ryser and Marth, 1987a). This organism was only isolated from a single cheese type produced using raw sheep milk.

TABLE 2. Incidence of *Listeria* spp. in milk and cheese

Sample	No of samples	% *Listeria* spp .	Species present		
			% *L.monocytogenes*	% *L.innocua*	% *L.ivanovii*
Raw cow milk	90	32.2 (29)*	8.9 (8)	21.1 (19)	2.2 (2)
Raw sheep milk	21	23.8 (5)	4.8 (1)	19 (4)	0 (0)
Raw goat milk	54	5.6 (3)	1.9 (1)	3.7 (2)	0 (0)
Cheese	305	4.9 (15)	1 (3)	3.9 (12)	0 (0)

* number of samples positive for *Listeria* spp. shown in parenthesis

TABLE 3. Incidence of *Listeria* spp. in raw milk* between June 1992 to May 1993

Month	Supplier	Isolated by Direct plating	Enrichment	Species
JUNE			**SHEEP MILK**	
	S-3	1 cfu/ml	(+)	*L. innocua*
			COW MILK	
	D-4	(-)	(+)	*L. innocua*
JULY			**SHEEP MILK**	
	S-3	1 cfu/ml	(+)	*L. innocua*
			COW MILK	
	D-3a	(-)	(+)	*L. monocytogenes*
	D-5a	(-)	(+)	*L. monocytogenes*
	D-5b	(-)	(+)	*L. innocua*
AUGUST			**GOAT MILK**	
	G-6	(-)	(+)	*L.monocytogenes*
			COW MILK	
	D-1	1 cfu/ml	(+)	*L. innocua*
	D-4	(-)	(+)	*L.monocytogenes*
SEPTEMBER			**SHEEP MILK**	
	S-3	(-)	(+)	*L.innocua*
			GOAT MILK	
	G-6	(-)	(+)	*L.innocua*
			COW MILK	
	D-3a	(-)	(+)	*L.ivanovii*
	D-4	(-)	(+)	*L.monocytogenes*
	D-5a	(-)	(+)	*L.monocytogenes*
OCTOBER			**SHEEP MILK**	
	S-3	(-)	(+)	*L.innocua*
			COW MILK	
	D-1	(-)	(+)	*L.ivanovii*
	D-4	(-)	(+)	*L.innocua*
	D-5a	(-)	(+)	*L.innocua*
	D-5b	(-)	(+)	*L.innocua*

*All other samples were *Listeria* spp. negative in 25 ml.

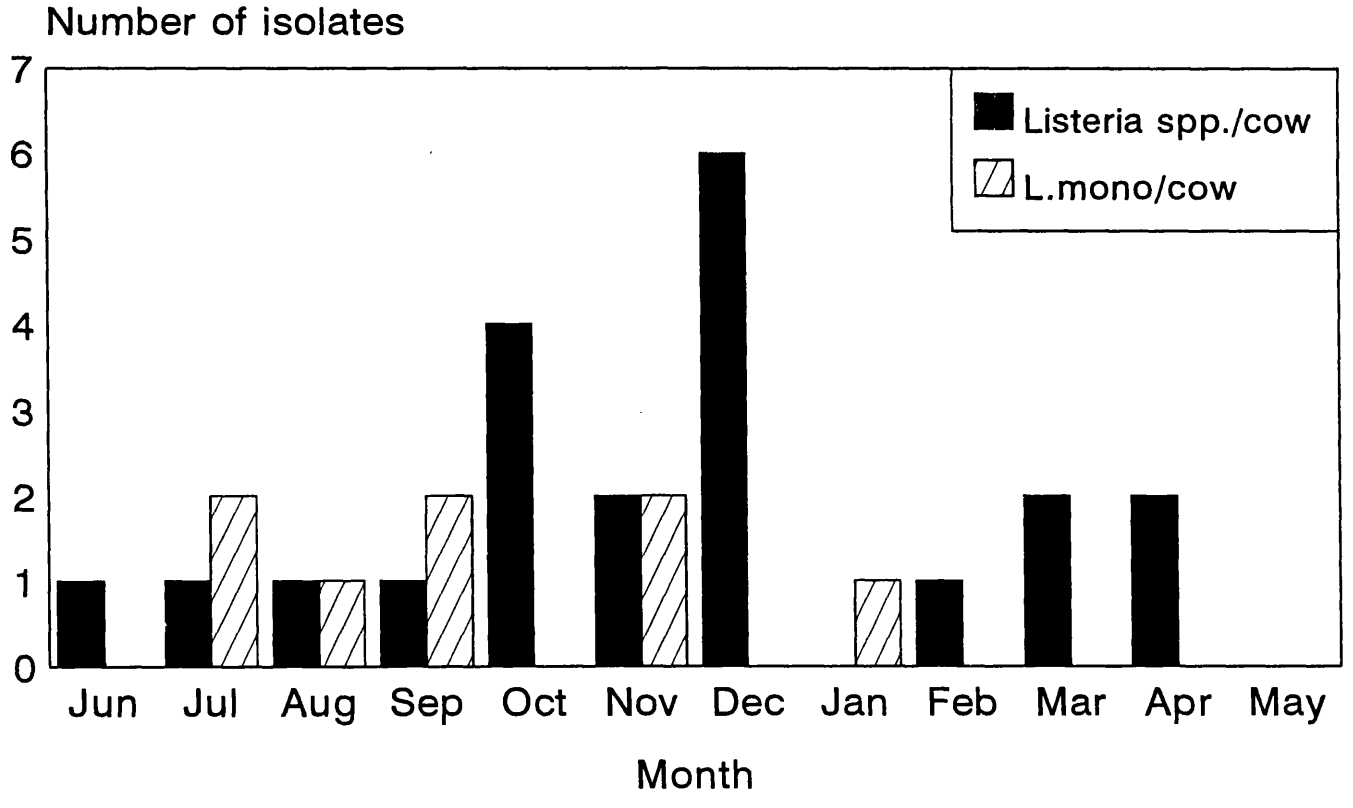
Continued

TABLE 3 continued. Incidence of *Listeria* spp. in raw milk* between June 1992 to May 1993

Month	Supplier	Isolation by Direct plating	Enrichment	Species
NOVEMBER			**COW MILK**	
	D-1	(-)	(+)	*L.innocua*
	D-2b	1 cfu/ml	(+)	*L.innocua*
	D-3a	(-)	(+)	*L.monocytogenes*
	D-3b	(-)	(+)	*L.monocytogenes*
DECEMBER			**COW MILK**	
	D-2a	(-)	(+)	*L.innocua*
	D-2b	(-)	(+)	*L.innocua*
	D-3a	(-)	(+)	*L.innocua*
	D-3b	(-)	(+)	*L.innocua*
	D-4	(-)	(+)	*L.innocua*
	D-5a	(-)	(+)	*L.innocua*
JANUARY			**COW MILK**	
	D-2a	(-)	(+)	*L.monocytogenes*
FEBRUARY			**COW MILK**	
	D-1	(-)	(+)	*L.innocua*
			GOAT MILK	
	G-7	(-)	(+)	*L. innocua*
MARCH			**COW MILK**	
	D-4	(-)	(+)	*L.innocua*
	D-5a	(-)	(+)	*L.innocua*
APRIL			**COW MILK**	
	D-2a	(-)	(+)	*L.innocua*
	D-2b	(-)	(+)	*L.innocua*
			SHEEP MILK	
	S-2	(-)	(+)	*L.monocytogenes*

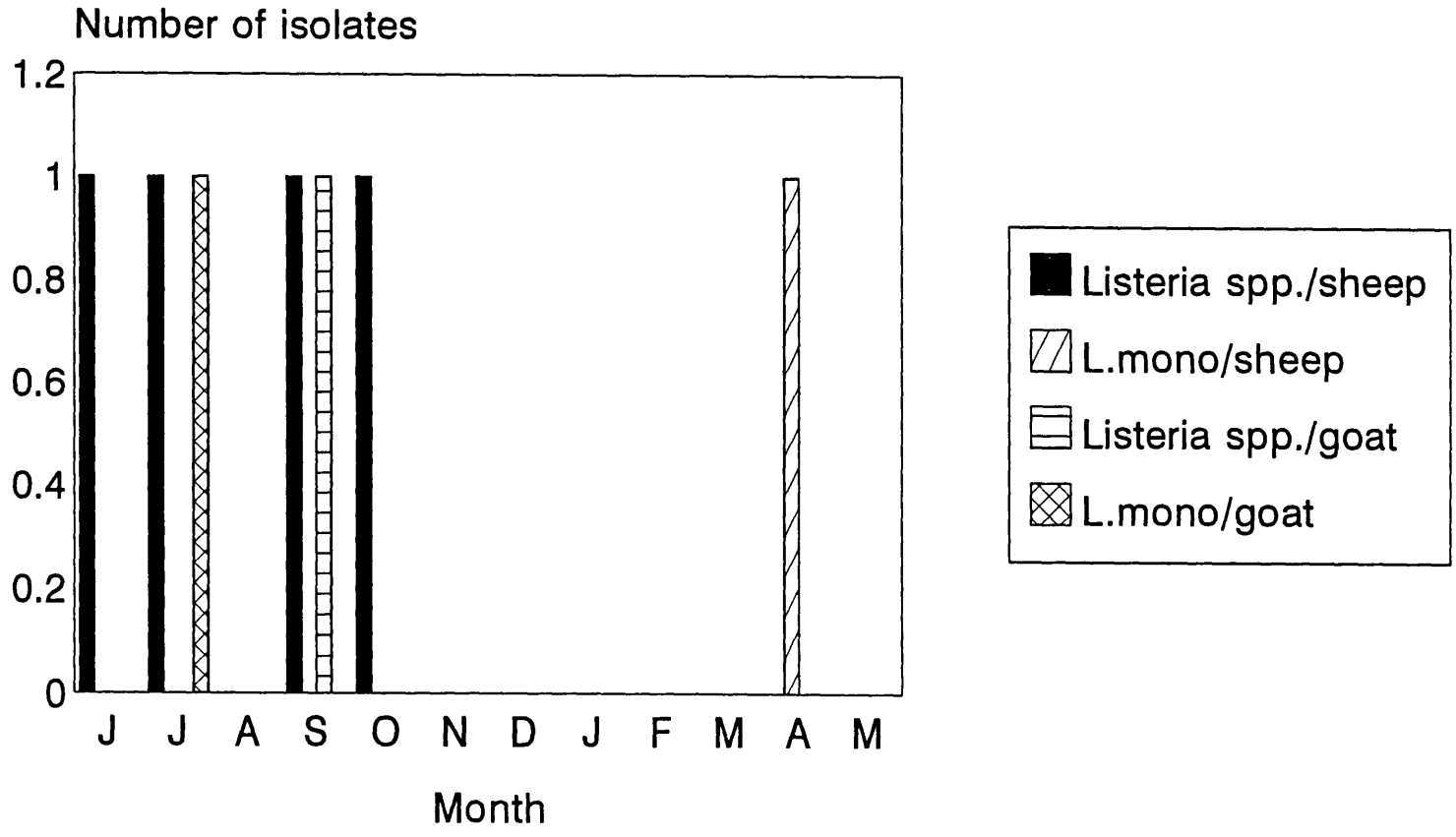
*All other samples were *Listeria* spp. negative in 25 ml.
No *Listeria* spp. were isolated from raw milk in May 1993

FIGURE 1. Incidence of Listeria monocytogenes and Listeria species* in raw cow milk between June 1993 - May 1994



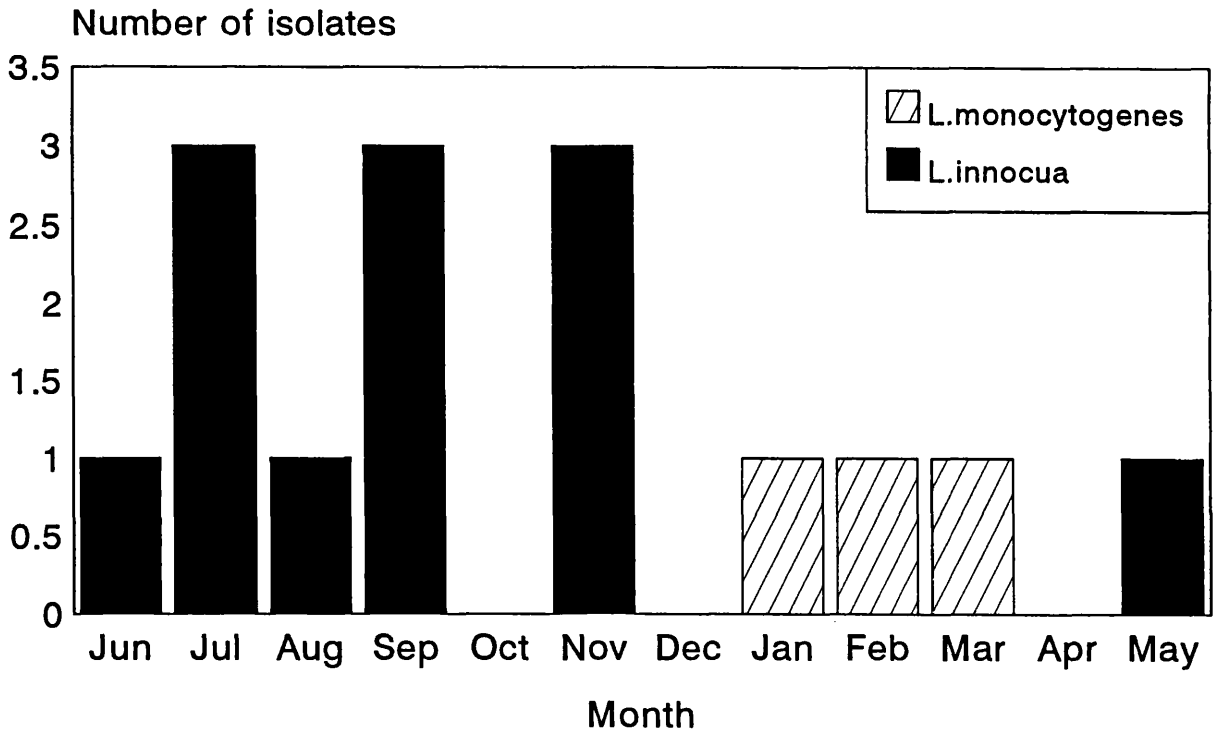
* excluding L.monocytogenes

FIGURE 2. Incidence of Listeria monocytogenes and Listeria species* in raw sheep and goat milks between June 1993 - May 1994



* excluding L.monocytogenes

FIGURE 3. Incidence of Listeria monocytogenes* and Listeria innocua in cheese between June 1993 - May 1994



*cheese type positive for L.monocytogenes was farm produced from unpasteurised sheep milk

TABLE 4. Incidence of *Listeria* spp. in cheese* between June 1992 to May 1993

Month	Cheese type (Supplier)	Isolation by Direct plating	Enrichment	Species
JUNE		**UNPASTEURISED SHEEP MILK CHEESE**		
	Semi-soft (S-3)	(-)	(+)	*L. innocua*
JULY		**UNPASTEURISED SHEEP MILK CHEESE**		
	Hard (S-1)	720 cfu/g	(+)	*L. innocua*
	Semi-soft (S-3)	775 cfu/g	(+)	*L. innocua*
		UNPASTEURISED COW MILK CHEESE		
	Half-fat Blue (C)	(-)	(+)	*L. innocua*
AUGUST		**UNPASTEURISED SHEEP MILK CHEESE**		
	Hard (S-1)	(-)	(+)	*L. innocua*
SEPTEMBER		**UNPASTEURISED SHEEP MILK CHEESE**		
	Hard (S-1)	10 cfu/g	(+)	*L. innocua*
	Roquefort (C)	(-)	(+)	*L. innocua*
		PASTEURISED COW MILK CHEESE		
	Cambozola (C)	(-)	(+)	*L. innocua*
NOVEMBER		**UNPASTEURISED SHEEP MILK CHEESE**		
	Hard (S-1)	1000 cfu/g	(+)	*L. innocua*
	Semi-soft (S-3)	280 cfu/g	(+)	*L. innocua*
		PASTEURISED COW MILK CHEESE		
	Dolcelatte (B)	90 cfu/g	(+)	*L. innocua*

*All other samples were *Listeria* spp. negative in 25 g cheese.
A-D = Retail supermarkets
S-1 and S-3 = Farm cheese manufacturers

Continued

TABLE 4 continued. Isolation of *Listeria* spp. from cheese* between June 1992 to May 1993

Month	Cheese type (Supplier)	Isolation by: Direct plating	Isolation by: Enrichment	Species
JANUARY		**UNPASTEURISED SHEEP MILK CHEESE**		
	Semi-soft (S-3)	10 cfu/g	(+)	*L.monocytogenes*
FEBRUARY		**UNPASTEURISED SHEEP MILK CHEESE**		
	Semi-soft (S-3)	140 cfu/g	(+)	*L.monocytogenes*
MARCH		**UNPASTEURISED SHEEP MILK CHEESE**		
	Semi-soft (S-3)	2560 cfu/g	(+)	*L.monocytogenes*
MAY		**UNPASTEURISED SHEEP MILK CHEESE**		
	Hard (S-1)	(-)	(+)	*L.innocua*

*All other samples were *Listeria* spp. negative in 25 g cheese.
A-D = Retail supermarkets
S-1 and S-3 = Farm cheese manufacturers

TABLE 5. Total bacterial count in raw sheep, cow and goat milks between June 1992 to May 1993

Supplier	Month (cfu/ml) JUNE	JULY	AUGUST	SEPTEMBER	OCTOBER	NOVEMBER
SHEEP MILK						
S-1	1.84×10^4	2.45×10^4	1.30×10^4	1.20×10^4	6.10×10^3	-
S-2	1.70×10^4	2.06×10^4	1.99×10^4	-	-	-
S-3	1.33×10^4	6.20×10^4	9.19×10^3	9.26×10^4	5.00×10^3	-
GOAT MILK						
G-1	-	4.63×10^5	8.52×10^4	-	-	-
G-2	-	-	8.70×10^4	-	-	-
G-3	5.74×10^3	1.06×10^4	3.49×10^3	-	-	-
G-4	8.27×10^3	-	-	-	-	-
G-5	-	4.63×10^5	1.04×10^3	6.30×10^4	1.65×10^5	-
G-6	4.07×10^4	2.24×10^5	4.96×10^4	1.11×10^5	1.90×10^5	-
G-7	7.79×10^3	2.22×10^4	3.74×10^3	1.46×10^4	1.20×10^5	-
G-8	5.41×10^3	2.13×10^4	9.09×10^3	5.03×10^3	8.78×10^3	-
G-9	4.72×10^3	-	3.03×10^4	6.90×10^3	4.54×10^4	-
G-10	7.12×10^4	-	1.11×10^5	5.00×10^4	4.17×10^4	-
COW MILK						
D-1	2.45×10^4	4.80×10^3	6.33×10^3	1.51×10^4	5.37×10^4	8.90×10^3
D-2a	1.87×10^3	3.82×10^3	8.61×10^4	6.24×10^4	1.85×10^4	2.30×10^4
D-2b	1.59×10^3	-	-	1.26×10^4	2.60×10^4	3.05×10^4
D-3a	1.40×10^4	2.33×10^4	5.09×10^4	5.56×10^4	4.45×10^4	7.40×10^4
D-3b	7.41×10^4	2.59×10^4	6.86×10^4	5.93×10^4	5.47×10^4	6.40×10^4
D-4	6.72×10^4	4.13×10^3	8.32×10^3	9.26×10^4	5.28×10^4	9.28×10^3
D-5a	4.53×10^4	9.72×10^4	1.84×10^4	3.43×10^4	1.70×10^4	1.14×10^5
D-5b	4.86×10^4	2.59×10^4	-	2.99×10^4	1.75×10^4	1.13×10^5

Continued

TABLE 5 continued. Total bacterial count in raw sheep, cow and goat milks between June 1992 to May 1993

Supplier	Month (cfu/ml) DECEMBER	JANUARY	FEBRUARY	MARCH	APRIL	MAY
SHEEP MILK						
S-1	-	-	-	-	2.96×10^4	2.03×10^2
S-2	-	-	-	7.24×10^3	5.33×10^3	3.37×10^3
S-3	-	-	-	9.74×10^5	3.21×10^4	2.39×10^4
GOAT MILK						
G-1	-	-	-	-	-	-
G-2	-	-	-	-	-	-
G-3	-	-	-	5.50×10^3	-	-
G-4	-	-	-	-	-	-
G-5	-	-	3.60×10^3	-	1.79×10^3	7.15×10^5
G-6	-	-	1.63×10^4	-	2.27×10^5	4.04×10^5
G-7	-	-	4.67×10^3	6.92×10^3	7.66×10^3	-
G-8	-	-	3.06×10^3	3.83×10^3	3.72×10^3	-
G-9	-	-	1.32×10^5	2.11×10^5	1.06×10^4	-
G-10	-	-	-	4.50×10^5	1.41×10^3	-
G-11	-	-	-	-	8.33×10^3	-
COW MILK						
D-1	1.40×10^4	2.34×10^4	2.14×10^4	2.76×10^4	1.99×10^4	2.08×10^4
D-2a	2.01×10^4	1.64×10^4	1.90×10^4	2.18×10^4	1.79×10^4	2.18×10^4
D-2b	1.61×10^4	1.88×10^4	2.36×10^4	2.98×10^4	5.33×10^3	2.23×10^4
D-3a	5.00×10^4	1.62×10^4	4.00×10^6	2.63×10^4	6.00×10^2	3.94×10^3
D-3b	4.44×10^4	1.40×10^4	5.10×10^6	2.00×10^4	1.63×10^4	3.25×10^3
D-4	1.70×10^4	1.50×10^4	1.97×10^4	1.68×10^4	6.89×10^3	1.99×10^4
D-5a	3.60×10^4	2.88×10^4	7.20×10^4	3.77×10^4	1.79×10^4	5.65×10^4
D-5b	3.51×10^4	3.00×10^4	-	-	1.79×10^4	6.39×10^4

TABLE 6. Total bacterial count in cheese between June 1992 to May 1993

Cheese type (Supplier)	Month (cfu/g) JUNE	JULY	AUGUST
UNPASTEURISED SHEEP MILK			
Hard (S-1)	2.41×10^7	4.05×10^7	1.64×10^7
Semi-soft (S-3)	1.73×10^8	7.69×10^7	1.09×10^8
Roquefort (C)	5.94×10^6	2.67×10^7	1.32×10^7
Roquefort (B)	2.24×10^6	6.11×10^7	2.28×10^6
PASTEURISED SHEEP MILK			
Feta (C)	2.97×10^4	3.11×10^4	2.53×10^4
Feta (B)	2.00×10^4	1.37×10^4	1.21×10^4
PASTEURISED GOAT MILK			
French Chevre (C)	1.48×10^6	1.24×10^8	2.54×10^5
English Goat (B)	1.05×10^8	-	-
Lingot Duberry (B)	0	0	0
UNPASTEURISED COW MILK			
Danish Blue (C)	7.62×10^5	1.08×10^6	6.61×10^5
Extra cream Dan. Blue (C)	3.25×10^5	1.40×10^6	1.14×10^6
Half fat Blue (C)	3.49×10^7	3.14×10^7	-
Danish Blue (D)	2.89×10^6	1.46×10^7	9.45×10^6
PASTEURISED COW MILK			
English Brie (C)	1.54×10^7	1.01×10^8	4.60×10^7
Cambazola (C)	1.60×10^6	2.03×10^4	3.56×10^5
Gorgonzola (C)	2.44×10^7	9.44×10^7	5.28×10^7
Dolcelatte (C)	9.98×10^5	4.78×10^7	9.74×10^6
Brie (D)	2.76×10^6	4.76×10^6	1.50×10^6
Cambozola (D)	-	-	8.13×10^4
Stilton (D)	1.09×10^8	6.50×10^7	-
Mini Roulade (D)	-	5.28×10^3	2.03×10^2
Brie (A)	8.61×10^7	4.67×10^6	2.03×10^4
Walnut Gateau (A)	9.15×10^2	5.04×10^6	3.65×10^5
Danish Blue (B)	1.62×10^5	3.96×10^5	1.63×10^5
Danish Mozzarella (B)	4.00×10^6	1.14×10^4	2.03×10^2
Brie (B)	2.43×10^7	1.15×10^8	3.91×10^7
Cambozola (B)	2.33×10^7	7.26×10^6	4.37×10^3
Dolcelatte (B)	1.14×10^7	6.31×10^7	4.27×10^7

A-D = Retail supermarkets
S-1 and S-3 = Farm cheese manufactures

Continued

TABLE 6 continued. Total bacterial count in cheese between June 1992 to May 1993

Cheese type	Month (cfu/g) SEPTEMBER	OCTOBER	NOVEMBER
UNPASTEURISED SHEEP MILK			
Hard (S-1)	1.61×10^7	1.45×10^7	3.55×10^7
Semi-soft(S-3)	9.35×10^7	1.90×10^8	5.65×10^7
Roquefort (C)	6.83×10^7	1.80×10^7	2.54×10^5
Roquefort (B)	3.84×10^7	6.44×10^6	3.47×10^7
PASTEURISED SHEEP MILK			
Feta (C)	9.08×10^6	1.14×10^6	3.00×10^4
Feta (B)	7.80×10^4	4.62×10^4	8.33×10^4
PASTEURISED GOAT MILK			
French Chevre (C)	7.55×10^7	5.10×10^3	3.15×10^6
English Goat (B)	-	2.55×10^7	8.70×10^6
Lingot Duberry (B)	0	0	0
UNPASTEURISED COW MILK			
Danish Blue (C)	4.37×10^5	9.47×10^6	1.71×10^6
Extra cream Dan. Blue (C)	3.36×10^7	1.73×10^5	-
Half fat Blue (C)	-	-	-
Danish Blue (D)	1.14×10^6	6.50×10^5	2.00×10^7
PASTEURISED COW MILK			
English Brie (C)	-	5.75×10^7	1.63×10^6
Cambazola (C)	1.64×10^7	7.22×10^6	4.93×10^4
Gorgonzola (C)	4.93×10^7	2.65×10^7	4.26×10^5
Dolcelatte (C)	9.52×10^6	1.14×10^7	1.72×10^7
Brie (D)	1.16×10^7	1.63×10^6	3.50×10^6
Cambozola (D)	1.10×10^8	1.35×10^7	-
Stilton (D)	6.90×10^7	2.25×10^7	5.04×10^7
Mini Roulade (D)	2.86×10^5	-	3.05×10^2
Brie (A)	5.47×10^7	4.60×10^7	4.88×10^5
Walnut Gateau (A)	8.10×10^6	3.86×10^4	4.78×10^5
Danish Blue (B)	-	8.60×10^5	6.44×10^6
Danish Mozzarella (B)	3.98×10^5	-	-
Brie (B)	1.39×10^8	4.88×10^5	7.86×10^6
Cambozola (B)	1.22×10^8	1.42×10^5	7.11×10^4
Dolcelatte (B)	3.90×10^7	1.25×10^6	2.58×10^7

A-D = Retail supermarkets
S1 and S3 = Farm cheese manufactures

Continued

TABLE 6 continued. Total bacterial count in cheese between June 1992 to May 1993

Cheese type	Month (cfu/g) DECEMBER	JANUARY	FEBRUARY
UNPASTEURISED SHEEP MILK			
Hard (S-1)	-	2.84×10^7	2.57×10^7
Semi-soft (S-3)	-	4.93×10^7	7.60×10^7
Roquefort (C)	2.50×10^7	1.48×10^7	6.90×10^7
Roquefort (B)	2.30×10^6	1.06×10^7	2.00×10^7
PASTEURISED SHEEP MILK			
Feta (C)	2.71×10^4	7.40×10^7	2.80×10^7
Feta (B)	2.20×10^4	4.73×10^7	8.40×10^4
PASTEURISED GOAT MILK			
French Chevre (C)	5.61×10^5	8.33×10^5	5.39×10^6
English Goat (B)	3.30×10^6	3.34×10^7	4.17×10^7
Lingot Duberry (B)	0	0	0
UNPASTEURISED COW MILK			
Danish Blue (C)	1.25×10^5	3.48×10^7	4.67×10^6
Extra cream Dan. Blue (C)	6.20×10^5	2.32×10^6	7.17×10^6
Half fat Blue (C)	-	-	-
Danish Blue (D)	1.65×10^7	2.83×10^7	4.03×10^7
PASTEURISED COW MILK			
English Brie (C)	5.60×10^7	2.24×10^8	5.43×10^7
Cambazola (C)	6.30×10^5	3.56×10^7	-
Gorgonzola (C)	1.60×10^5	9.90×10^7	2.70×10^5
Dolcelatte (C)	8.00×10^5	1.13×10^8	4.77×10^7
Brie (D)	4.00×10^4	4.33×10^6	1.10×10^6
Cambozola (D)	1.00×10^5	3.32×10^6	1.25×10^8
Stilton (D)	1.00×10^8	1.49×10^8	1.83×10^7
Mini Roulade (D)	4.00×10^2	1.40×10^2	1.60×10^4
Brie (A)	7.95×10^7	6.28×10^8	4.40×10^7
Walnut Gateau (A)	7.10×10^2	2.19×10^8	2.06×10^7
Danish Blue (B)	1.40×10^6	1.44×10^7	3.50×10^7
Danish Mozzarella (B)	8.92×10^6	1.14×10^6	2.00×10^6
Brie (B)	1.15×10^8	1.86×10^8	6.90×10^7
Cambozola (B)	-	8.30×10^7	6.20×10^7
Dolcelatte (B)	8.60×10^6	3.18×10^8	7.90×10^7

A-D = Retail supermarkets
S-1 and S-3 = Farm cheese manufactures

Continued

TABLE 6 continued. Total bacterial count in cheese between June 1992 to May 1993

Cheese type	Month (cfu/g) MARCH	APRIL	MAY
UNPASTEURISED SHEEP MILK			
Hard (S-1)	9.00×10^7	4.42×10^7	7.33×10^6
Semi-soft (S-3)	8.33×10^4	6.48×10^8	2.07×10^6
Roquefort (C)	2.17×10^6	6.06×10^7	1.86×10^7
Roquefort (B)	1.60×10^7	6.35×10^6	1.51×10^6
PASTEURISED SHEEP MILK			
Feta (C)	3.26×10^8	4.13×10^4	2.15×10^4
Feta (B)	3.52×10^8	1.80×10^4	6.50×10^3
PASTEURISED GOAT MILK			
French Chevre (C)	4.20×10^2	2.03×10^2	6.10×10^2
English Goat (B)	8.60×10^7	2.70×10^8	8.23×10^5
Lingot Duberry (B)	0	0	0
UNPASTEURISED COW MILK			
Danish Blue (C)	1.02×10^8	1.16×10^8	2.42×10^7
Extra cream Dan. Blue (C)	2.08×10^7	5.07×10^6	8.90×10^6
Half fat Blue (C)	-	-	-
Danish Blue (D)	1.28×10^8	8.06×10^7	7.58×10^6
PASTEURISED COW MILK			
English Brie (C)	1.24×10^8	9.35×10^7	3.23×10^8
Cambazola (C)	2.17×10^7	7.99×10^4	5.08×10^5
Gorgonzola (C)	4.78×10^8	9.78×10^7	1.39×10^8
Dolcelatte (C)	9.60×10^7	1.26×10^8	5.74×10^7
Brie (D)	4.33×10^6	6.50×10^5	2.03×10^6
Cambozola (D)	3.15×10^8	-	-
Stilton (D)	-	2.07×10^8	4.71×10^7
Mini Roulade (D)	5.00×10^3	4.67×10^3	1.63×10^3
Brie (A)	9.20×10^7	5.74×10^6	8.07×10^6
Walnut Gateau (A)	6.67×10^3	2.24×10^5	2.03×10^4
Danish Blue (B)	2.56×10^7	4.07×10^7	6.35×10^6
Danish Mozzarella (B)	2.88×10^5	3.51×10^8	1.02×10^2
Brie (B)	5.93×10^7	9.52×10^7	8.64×10^6
Cambozola (B)	1.73×10^8	2.07×10^6	1.30×10^6
Dolcelatte (B)	1.68×10^7	1.29×10^7	1.00×10^7

A-D = Retail supermarkets
S-1 and S-3 = Farm cheese manufacturers

CHAPTER 4

USE OF IMPEDANCE TECHNOLOGY TO DETECT CHEESE RIPENING MOULDS AND CHEESE STARTER CULTURES WHICH MODULATE THE GROWTH OF *LISTERIA* SPECIES

4.1 INTRODUCTION

In 1986 approximately 60% of French Brie cheese was recalled from the American market because of contamination with *L.monocytogenes* (Anon 1986). This prompted concern about survival and growth of this organism in soft cheese. A large proportion of soft and semi-soft cheeses are mould ripened, mostly using *Penicillium roqueforti* for blue-veined cheese and *Penicillium camemberti* for white mould cheese.

The growth kinetics of *L.monocytogenes* has been examined in many cheeses (Ryser and Marth 1987a, Ryser and Marth 1987b, Papageorgiou and Marth 1989 and Dominguez *et al.* 1987) and it has been found to have grown to high numbers or to be maintained during ripening in soft-cheeses such as Camembert. Others have reported that starter culture metabolism can influence the growth of *L.monocytogenes in vitro* (Raccach *et al.*, 1989; Wenzel and Marth, 1991). Ryser and Marth (1988) showed the generation time of *L.monocytogenes* at 6°C to be quicker in Camembert cheese whey previously cultured with *Penicillium camemberti* than in uncultured whey. So far this is the only report on the influence of cheese ripening moulds on the growth of *Listeria* spp.

This work investigated whether *Penicillium roqueforti* and *Penicillium camemberti* cheese mould strains and cheese lactic acid starter culture strains

could influence the growth of *Listeria* spp. Interactive effects were studied using impedance technology.

4.2 EXPERIMENTAL PROCEDURES

4.2.1 CULTURES

Five strains of *L.monocytogenes* used in this study were SITC 12/1A, SITC 404/2, NCTC 7973, MMB 7 and 8, two strains each of *L.innocua*, SITC 112/2A/6 and NCTC 11288 and *L.murrayi*, NCTC 10812 and MMB 4 (see section 2.1.1).

Six *Penicillium roqueforti* strains used in this study were IMI strains 148775, 24313 and 129207 and commercial strains from Lacto. Labo (Groupe Rhône-Poulenc) PRB2 and PRB6 and Centro Sperimentale Del Latte strain NO-N (see section 2.1.1). Five *Penicillium camemberti* strains used in this study were IMI strains 129208, 27831 and 214010 and commercial strains from Lacto. Labo namely, Niege and Sam 2 (see section 2.1.1).

Three *Lactococcus lactis* strains used in this study were E9275, SL391 and SL213 from Marschall-Eurozyme U.K. (Rhône-Poulenc) (see section 2.1.1).

4.2.2 INTERACTION OF *LISTERIA* SPP. WITH CHEESE RIPENING MOULDS

Fungal strains were streaked onto MA (see section 2.1.4) and incubated at 30°C for 7 days or until growth was satisfactory (i.e. fungal growth all over the agar). 10 ml TSB (see section 2.1.4) was inoculated with a pure culture of the mould and incubated at 30°C for 7 days. A 1% inoculum from this culture was then added into 30 ml TSB and and incubated at 30°C for 7 days. A 1% inoculum of this broth was added to 300 ml TSB and incubated at 30°C for 7

days, shaken at 100 xg (G24 Environmental Incubator, New Brunswick Scientific, USA). The medium was centrifuged at 18,000 xg for 30 min and the supernatant fluid aseptically removed and filter sterilised through 0.2μm pore size membrane filters (Sartorius). The resulting sterile spent broth supernatant was stored at 4°C until used.

One ml of either the spent broth supernatant from each fungal culture (diluted with fresh TSB in a ratio of 1:1 for use in assay) or fresh TSB (control) was added to duplicate module wells and inoculated with a culture of a single *Listeria* spp. to give a starting inoculum level of 10 cfu/ml. The modules were incubated at 30°C for 50 h and the change in impedance measured as capacitance of the media was monitored using a Bactometer M64 (BioMérieux UK Ltd, Basingstoke) (see section 2.2.2). A detection time (dt) in hours was given once the *Listeria* spp. reached a defined level of $1x10^6$ cfu/ml inside the module well.

4.2.3 INTERACTION OF *LISTERIA SPP.* WITH CHEESE STARTER CULTURES

The *Lactococcus lactis* strains were streaked onto TSYEA (see section 2.1.4) and incubated at 37°C for 18 h. 2x10 ml TSB were inoculated with a pure culture of the bacterium and incubated at 37°C for 18 h. The medium was centrifuged at 3,500 xg for 30 min and the supernatant fluid aseptically removed and filter sterilised through 0.2μm pore size membrane filters (Sartorius). The resulting sterile spent broth supernatant was stored at 4°C until used.

One ml of either the spent broth supernatant from each bacterial culture

(diluted with fresh TSB in a ratio of 1:1 for use in assay) or fresh TSB (control) was added to duplicate module wells and inoculated with a culture of a single *Listeria* spp. to give a starting inoculum level of 10 cfu/ml. The modules were incubated at 30°C for 50 h and the change in impedance measured as capacitance of the media was monitored using a Bactometer M64 (BioMérieux UK Ltd, Basingstoke, Hants) (see section 2.2.2). A detection time (dt) in hours was given once the *Listeria* spp. reached a defined level of $1x10^6$ cfu/ml inside the module well.

4.2.4 STATISTICAL ANALYSIS

All statistical analysis was carried out using Analyis of Variance (see section 2.2.11).

4.3 RESULTS

The dt for growth of *Listeria* spp. in spent sterile broth supernatants which had supported growth of *P.roqueforti* strains were compared with the dt in control broths and are outlined in Tables 7-9. All results are the mean of duplicate tests repeated on three separate occasions and were subjected to Analysis of Variance (see section 2.2.11). It was found that *P.roqueforti* strains IMI 24313, PRB2 and NO-N had a significant ($P<0.05$) inhibitory effect on all the *Listeria* spp. used. With these *P.roqueforti* strains, *L.murrayi* (NCTC 10812) was so greatly inhibited that no detection time was recorded after 50 hours. The remaining *P.roqueforti* strains IMI 148775, IMI 129207 and PRB6, enhanced the growth of all the *Listeria* spp. significantly ($P<0.05$).

The dt for growth of *Listeria* spp. in spent sterile broth supernatants which had supported growth of *P.camemberti* strains were compared with growth in control broths and are outlined in Tables 10-12. All results are the mean of duplicate tests repeated on three seperate occasions and were subjected to Analysis of Variance (see section 2.2.11). It was found that only the commercial *P.camemberti* strain NIEGE had a significant ($P < 0.001$) inhibitory effect on all the *Listeria* spp. studied. Both strains of *L.murrayi* (NCTC 10812 and MMB 4) were inhibited to a greater extent than any other *Listeria* spp., followed closely by *L.monocytogenes* (SITC 404/2). The remaining *P.camemberti* strains, which include IMI 214070, IMI 129208, IMI 127831 and a commercial strain SAM 2, all had no statistically significant effect on the growth of *Listeria* spp.

The dt for growth of *L.monocytogenes* and *L.innocua* in spent sterile broth supernatants which had supported growth of *Lactococcus lactis* strains were compared with the dt in control broths (Table 13). *Lactococcus lactis* strain E 9275 was significantly ($p < 0.05$) inhibitory towards all the *Listeria* spp. strains tested. *Lactococcus lactis* strains SL 213 and SL 391 were not significantly inhibitory towards the *Listeria* spp. test strains and gave similar results as the control TSB.

4.4 DISCUSSION

Sterile spent broth supernatants which had previously supported growth of fungal cultures were used throughout this study. The reasoning behind this was to try and duplicate the growth effects the fungal cultures produce during ripening of cheese and collect any byproducts normally released into the cheese

during this ripening. The spent supernatant broth was then sterilised and diluted with fresh sterile TSB in a 1:1 ratio. The fresh TSB was added to provide nutrients for growth of the *Listeria* spp. in the impedance experiments. Any changes in the spent supernatant broths (resulting from the metabolism of the fungal cultures only) could then be shown.

Lactococcus lactis strains E 9275, SL 213 and SL 391 are all blended together to produce a direct vat inoculation starter culture for cheese manufacture. Since this is a commercial product, the percentage of each strain used in the mixed inoculum is unknown. It is also unknown whether *Lactococcus lactis* E 9275 could be used as a single strain starter culture in cheese manufacture since this strain had a significant ($p < 0.05$) inhibitory effect on the growth of the *Listeria* spp. strains tested. Sulzer and Busse (1991) showed that *Lactococcus lactis* strain 1881 along with a commercial mixed starter culture had no effect on the growth of *L.monocytogenes* (SLCC 1694) during manufacture and ripening of Camembert cheese but completely inhibited the *L.monocytogenes* (SLCC 1694) strain when used as the sole starter culture. Raccach *et al* (1989) showed that four of the five strains of lactic acid bacteria most antagonistic towards *L.monocytogenes* were known to produce bacteriocins.

Of the five *L.monocytogenes* strains studied, SITC 404/2 was affected the most by the fungal strains *in vitro* (Tables 7-12). All these results show that not all *P. roqueforti*, *P. camemberti* and *Lactococcus lactis* strains affect the growth of *Listeria* spp. *in vitro*. These results indicate that selection of *Listeria* inhibitor strains of fungal cultures and cheese starter cultures could affect the

overall growth of *Listeria* spp., in particular *L.monocytogenes*, in cheese resulting in possible safer manufactured mould-ripened cheese. Proteolysis, lipolysis and resultant production of free fatty acids in blue-veined cheese are very high compared with other cheese varieties due to *P.roqueforti* metabolism. Methyl ketones are formed via the fatty acid β-oxidation pathway (Law, 1984). The intensity of the biochemical activity of *P.roqueforti* varies considerably among strains and the choice of strain has a major effect on the quality of blue-veined cheese. Differences in the biochemical activity between strains of *P.roqueforti* could also have an effect on the type of flora present in the final cheese. Less variation between the biochemical activity of *Penicillium camemberti* strains have been shown (Law, 1984). The fungal and starter cultures found to inhibit the growth of *L.monocytogenes* could only be used if they gave desirable starter and ripening activities required for satisfactory cheese production, including correct flavour balance and texture.

TABLE 7. Effect of *Penicillium roqueforti* IMI 148775, 24313 and 129207 strains on growth of *Listeria* spp.

Species	Listeria strain	Detection time (hours)[1]			
		TSB[2]	TSB 148775[3]	TSB 24313[3]	TSB 129207[3]
L. monocytogenes	SITC 12/1A	11.93	11.32	12.48	10.42
L. monocytogenes	NCTC 7973	16.98	14.92	18.59	13.82
L. monocytogenes	SITC 404/2	12.35	11.30	14.32	11.58
L. monocytogenes	MMB 7	11.42	11.22	14.83	11.25
L. monocytogenes	MMB 8	12.80	11.52	15.63	11.65
L. innocua	NCTC 11288	12.85	11.37	14.10	11.37
L. innocua	SITC 112/ 2A/6	11.55	10.45	13.18	10.75
L. murrayi	MMB 4	12.15	11.15	*	11.65
L. murrayi	NCTC 10812	13.35	12.10	*	13.20

[1]Mean of 3 experiments done in duplicate.
[2]Tryptone soya broth.
[3]Tryptone soya broth that had supported growth of *P. roqueforti* IMI 148775 or IMI 24313 or IMI 129207 and was diluted 1:1 with fresh TSB.
*No growth detected up to 50 hours.
S.E.M. = 0.06 (D.F. = 180)

TABLE 8. Effect of commercial *Penicillium roqueforti* PRB2 and PRB6 strains on growth of *Listeria* spp.

Species	Listeria strain	Detection time (hours)[1] TSB[2]	TSB PRB2[3]	TSB PRB6[3]
L. monocytogenes	SITC 12/1A	10.55	11.25	10.00
L. monocytogenes	SITC 404/2	12.43	26.98	11.95
L. innocua	SITC 236/2/8	11.20	11.15	10.38
L. innocua	SITC 112/2A/6	11.05	11.98	10.78
L. murrayi	NCTC 10812	12.60	*	11.63

[1]Mean of 3 experiments done in duplicate.
[2]Tryptone soya broth.
[3]Tryptone soya broth that had supported growth of *P. roqueforti* Lacto. Lab PRB2 or PRB6 and was diluted 1:1 with fresh TSB.
*No growth detected up to 50 hours.
S.E.M.=0.07 (D.F.=75)

TABLE 9. Effect of commercial *Penicillium roqueforti* NO-N strain on growth of *Listeria* spp.

Species	Listeria strain	Detection time (hours)[1] TSB[2]	TSB NO-N[3]
L. monocytogenes	SITC 12/1A	11.48	12.23
L. monocytogenes	SITC 404/2	12.73	13.13
L. monocytogenes	NCTC 7973	11.70	15.40
L. innocua	SITC 236/2/8	11.15	12.18
L. innocua	SITC 112/2A/6	12.20	12.70
L. murrayi	NCTC 10812	12.65	*

[1]Mean of 3experiments done in duplicate.
[2]Tryptone soya broth.
[3]Tryptone soya broth that had supported growth of *P. roqueforti* Centro Sperimentale Del Latte NO-N and was diluted 1:1 with fresh TSB
*No growth detected up to 50 hours.
S.E.M.=0.235 (D.F.=60)

TABLE 10. Effect of *Penicillium camemberti* IMI 27831 and 12908 strains on growth of *Listeria* spp.

Species	Listeria strain	Detection time (hours)[1]		
		TSB[2]	TSB 27831[3]	TSB 12908[3]
L. monocytogenes	SITC 12/1A	15.28	14.32	14.78
L. monocytogenes	NCTC 7973	23.93	21.63	21.75
L. monocytogenes	SITC 404/2	16.53	15.97	17.02
L. monocytogenes	MMB 7	18.32	16.28	16.97
L. monocytogenes	MMB 8	16.87	16.08	16.42
L. innocua	NCTC 11288	15.20	15.93	15.82
L. innocua	SITC 112/ 2A/6	16.35	15.30	16.02
L. murrayi	MMB 4	17.87	17.38	20.02
L. murrayi	NCTC 10812	15.80	16.35	16.38

[1]Mean of 3 experiments done in duplicate.
[2]Tryptone soya broth.
[3]Tryptone soya broth that had supported growth of *P. camemberti* IMI 27831 or IMI 129208 and was diluted 1:1 with fresh TSB.
S.E.M. = 0.648 (D.F. = 135)

TABLE 11. Effect of *Penicillium camemberti* IMI 214070 strain on growth of *Listeria* spp.

Species	Listeria strain	Detection time (hours)[1] TSB[2]	TSB 214070[3]
L. monocytogenes	SITC 12/1A	14.00	14.15
L. monocytogenes	NCTC 7973	22.15	20.18
L. monocytogenes	SITC 404/2	16.38	17.03
L. monocytogenes	MMB 7	16.32	16.30
L. monocytogenes	MMB 8	15.40	14.78
L. innocua	NCTC 11288	13.58	13.93
L. innocua	SITC 112/ 2A/6	15.13	15.03
L. murrayi	MMB 4	15.48	15.87
L. murrayi	NCTC 10812	15.98	15.58

[1]Mean of 3 experiments done in duplicate.
[2]Tryptone soya broth.
[3]Tryptone soya broth that had supported growth of *P. camemberti* IMI214070 and was diluted 1:1 with fresh TSB.
S.E.M. = 1.038 (D.F. = 90)

TABLE 12. Effect of commercial *Penicillium camemberti* SAM2 and NIEGE strains on growth of *Listeria* spp.

Species	Listeria strain	Detection time (hours)[1] TSB[2]	TSB SAM2[3]	TSB NIEGE[3]
L. monocytogenes	SITC 12/1A	11.73	11.73	13.40
L. monocytogenes	NCTC 7973	20.15	17.13	21.93
L. monocytogenes	SITC 404/2	14.35	13.69	22.63
L. monocytogenes	MMB 7	12.95	13.48	16.82
L. monocytogenes	MMB 8	13.67	12.83	18.67
L. innocua	NCTC 11288	13.22	12.90	13.20
L. innocua	SITC 112/ 2A/6	11.93	11.65	15.25
L. murrayi	MMB 4	12.12	11.90	27.35
L. murrayi	NCTC 10812	13.70	13.50	28.37

[1]Mean of 3 experiments done in duplicate.
[2]Tryptone soya broth.
[3]Tryptone soya broth that had supported growth of *P. camemberti* Lacto.Lab SAM2 or NIEGE and was diluted 1:1 with fresh TSB.
S.E.M.=2.141 (D.F.=135)

TABLE 13. Effect of Lactic acid starter cultures on growth of *Listeria* spp.

Species	Listeria strain	Detection time (hours)[1] TSB[2]	TSB E9275[3]	TSB SL213[3]	TSB SL391[3]
L. monocytogenes	SITC 12/1A	14.70	17.00	14.70	15.70
L. monocytogenes	NCTC 7973	21.60	23.80	20.40	20.20
L. monocytogenes	SITC 404/2	15.60	17.90	15.30	15.80
L. monocytogenes	MMB 7	15.90	18.90	16.00	16.90
L. monocytogenes	MMB 8	15.90	18.70	15.70	16.70
L. innocua	NCTC 11288	15.80	16.60	15.90	16.40
L. innocua	SITC 112/ 2A/6	15.50	18.00	15.50	16.10

[1]Mean of 3 experiments done in duplicate.
[2]Tryptone soya broth.
[3]Tryptone soya broth that had supported growth of *Lactococcus lactic* E9275 or SL213 or SL391 and was diluted 1:1 with fresh TSB.
*No growth detected up to 50 hours.
S.E.M. = 0.368 (D.F. = 140)

CHAPTER 5

SURVIVAL AND GROWTH OF *LISTERIA MONOCYTOGENES* IN SOFT AND SEMI-SOFT MANUFACTURED CHEESE

5.1 INTRODUCTION

Listeria monocytogenes is a motile, Gram-postive bacterium widely distributed in the environment (Welshimer and Donker-Voet 1971). It can grow over a wide range of temperature, pH, osmotic pressure and since it is a pyschrotroph, it is capable of growth at 4°C (refrigeration temperatures).

Results from Chapter 3 show that *Listeria monocytogenes* can be isolated from semi-soft mould ripened cheese. Results from impedance studies (Chapter 4) have shown that factors in culture supernatants from different strains of *Penicillium roqueforti* can significantly affect the growth of *L.monocytogenes* by either enhancing or partially inhibiting the growth of this organism. It must be noted that complete inhibition of growth of *L.monocytogenes* was never demonstrated but rather a reduction in growth rate was shown with these fungal metabolites. Only one strain of *P.camemberti* showed the ability to inhibit growth of *L.monocytogenes*, the four remaining *P.camemberti* strains had no effect on growth.

This work investigated whether *P.roqueforti* and *P.camemberti* cheese mould strains could influence the growth of *L.monocytogenes* (as previously shown using impedance technology) when used to produce a mould-ripened soft cheese. The effect on the growth and survival of *L.monocytogenes* at low levels (1 and 10 cfu/ml) in Camembert cheese made from raw cow milk was also studied. Further, post-manufacture contamination of Camembert cheese (two

weeks after manufacture) with *L.monocytogenes* was also studied.

5.2 EXPERIMENTAL PROCEDURES

5.2.1 CULTURES

Listeria monocytogenes strain SITC 404/2 was used in these experiments since this strain showed greatest modulation in growth due to the influence of fungal spent broth supernatants (see section 2.1.1). Six *Penicillium roqueforti* strains used in this study were IMI strains 148775, 24313 and 129207 and commercial strains from Lacto. Lab PRB2 and PRB6 and Centro Sperimentale Del Latte strain NO-N (see section 2.1.1). Five *Penicillium camemberti* strains also used were IMI strains 129208, 27831 and 214010 and two commercial strains from Lacto. Lab namely, Niege and Sam 2 (see Section 2.1.1). All cultures were maintained on Czapek Agar (see section 2.1.2).

Listeria monocytogenes used to inoculate milk for manufacture of Blue-veined and Camembert cheese was added to raw milk in the cheese vat to yield approximately $3x10^4$ cfu/ml of milk. This level was selected so that any effect on the growth of *L.monocytogenes* in the cheese due to the presence of the fungal strain could be readily detected by direct plating of the cheese sample.

5.2.2 MANUFACTURE OF BLUE-VEINED CHEESE

Fungal strains found to modulate the growth of *L.monocytogenes* in impedance studies (see Chapter 4) were selected for use in laboratory scale cheese manufacture.

The cheese was produced by the method outlined by Scott (1986) for Caledonian Blue cheese (Scottish Agricultural College, Auchincruive, Scotland) using raw sheep milk. A 24 h TSB culture of *L.monocytogenes* was used to inoculate milk for cheese manufacture and added to raw milk (10 litres) in the cheese vat to yield approximately $3x10^4$ cfu/ml of milk. *Penicillium roqueforti* strains used in the manufacture of Blue-veined cheese were added to raw milk in the cheese vat to yield about $8.25x10^6$ spores per ml. A commercial mixed-strain starter culture was added to raw milk in the cheese vat at the start of cheese manufacture (MA 400 EZAL1) (see section 2.1.1).

5.2.3 MANUFACTURE OF CAMEMBERT CHEESE

Fungal strains found to modulate the growth of *L.monocytogenes* in impedance studies (see Chapter 4) were selected for use in laboratory scale cheese manufacture.

The cheese was produced by the method outlined by Scott (1986) for traditional Camembert soft cheese (Modern Technology) using pasteurised and unpasteurised cow and goat milks. An APV Junior heat exchanger system modified to heat samples to 140°C (Junior Paraflow, APV Baker, Derby) was used to pasteurise milk when required. It was set at 71.7°C with a holding time of 15 s. This was maintained by the insertion of a tube of appropriate length in the holding section with a production flow of 90 l/h.

A 24 h TSB culture of *L.monocytogenes* was used to inoculate milk for cheese manufacture and added to raw milk in vat to yield approximately $3x10^4$ cfu/ml of milk. *Penicillium camemberti* strains used in the manufacture of

Camembert cheese were added to raw milk (10 litres) in the cheese vat to yield approximately $3.45x10^8$ spores per ml. A commercial mixed-strain starter culture was added to raw milk in the cheese vat at the start of cheese manufacture (MA 400 EZAL1) (see section 2.1.1).

5.2.4 ENUMERATION OF *L.MONOCYTOGENES*

Appropriate dilutions of raw milk, inoculated milk and whey were directly spread plated (Stainer *et al.*, 1987) (2x500µl) onto Oxford agar (see section 2.1.4) to enumerate *L.monocytogenes.* Samples for bacteriological analysis were taken from the 24 hr curd and whey and a wedge was aseptically removed from the Camembert cheese every 7 days and from the Blue-veined cheese every 4 weeks until the cheese had ripened. 25g of the curd or cheese samples were added to 225 ml of LEB (see section 2.1.4) warmed to 45°C contained in sterile stomacher bag and blended for 2 min in a stomacher (Stomacher 400). Duplicate 2x500µl volumes of the enrichment broths and/or dilutions were directly plated onto the surface of Oxford agar so that well isolated colonies were obtained if present. The enrichment broths were then incubated at 30°C for 48 h and only plated if recovery of *L.monocytogenes* by direct plating was unsuccessful. The plates were incubated at 37°C for 48 h. Colonies typical of those formed by *L.monocytogenes* were counted, and selected colonies were confirmed as *L.monocytogenes* according to the International Dairy Federation Procedure 143 (Anon, 1990) (see section 2.2.3).

5.2.5 POST-MANUFACTURE CONTAMINATION OF CAMEMBERT CHEESE

The *Pencilliun camemberti* strains used were two commercial strains from Lacto. Lab namely, Niege and Sam 2 (see section 2.1.1). *L.monocytogenes* was inoculated into sterile distilled water at levels of 3 x 10^3 cfu/ml (runs 1 and 2) and 1 x 10^5 cfu/ml (run 3). Two week old Camembert cheese (see section 5.2.3) was inoculated with *L.monocytogenes* by dipping each cheese into the inoculated water. These levels were selected so that any effect on the growth of *L.monocytogenes* on or in the cheese due to the presence of the fungal strain could be readily detected by direct plating of the cheese sample. Samples for bacteriological analysis (10g) were taken from the inside and outside of the cheese every 7 days until the cheese had ripened. 10g of the cheese sample was added to 90 ml of LEB and enumeration and isolation of *L.monocytogenes* carried out as before (see section 2.2.3).

5.3 RESULTS

5.3.1 GROWTH OF *L.MONOCYTOGENES* IN BLUE-VEINED CHEESE

Results of the survival of *L.monocytogenes* in blue-veined cheese are in Table 14. These are the results of three replicate trials using three different *P.roqueforti* strains. No *Listeria* spp. were present in the raw sheep milk used for cheese manufacture. The initial level of *L.monocytogenes* inoculated into ten litres of sheep milk was between 1x10^4 - 5x10^4 cfu/ml in the three trials and within 24 h the level of *L.monocytogenes* had increased to between 1.73x10^5 - 2.90x10^5 cfu/g in the cheese. This result is similar to those reported by Papageorgiou and Marth (1989) for *L.monocytogenes* in semi-hard blue-veined

cheese. No *L.monocytogenes* was isolated from the whey collected for up to 24 h after manufacture. This suggested that all the *L.monocytogenes* had been trapped inside the curd during manufacture.

Within 4 weeks of manufacture, *L.monocytogenes* could not be detected by direct plating (2x500µl) on Oxford agar, but could be detected using enrichment procedures. This was also the case with the 8 week samples. At 12, 16 and 20 weeks, no *L.monocytogenes* could be isolated using enrichment procedures. Papageorgiou and Marth (1989) also noted a sharp decrease of *L.monocytogenes* in blue-veined cheese over the first 50 days (roughly 6 weeks) of ripening and could no longer detect *L.monocytogenes* by direct plating at 110-120 days (roughly 16-17 weeks) but could detect *L.monocytogenes* after cold enrichment of these samples for 4-6 weeks.

The results of these trials showed that *L.monocytogenes* had grown during the first 24 h of manufacture, but thereafter decreased over the cold storage period until it could no longer be detected using the recommended IDF enrichment procedures for dairy products (Anon 1990).

Despite the findings *in vitro* (see Chapter 4), growth and survival of *L.monocytogenes* in the period up to 8 weeks post-manufacture was found to be unaltered by the *P.roqueforti* strain used in the production of blue-veined cheese.

5.3.2 GROWTH OF *L.MONOCYTOGENES* IN CAMEMBERT CHEESE

Results of the survival of *L.monocytogenes* in Camembert cheese are outlined in Figures 4-6. No *Listeria* spp. were present in the raw milk from

cow and goat herds used for cheese manufacture. It was found that Camembert cheese produced from pasteurised and unpasteurised cow and goat milk (Figures 4-6) supported the growth of an inoculum of *L.monocytogenes* during manufacture. *L.monocytogenes* continued to grow and survive in all the cheeses during ripening and reached levels as high as 8.91×10^7 cfu/g. The greatest increase in counts during manufacture was obtained with raw cow milk with roughly an increase of two logs while the lowest increase of less than one log was obtained using raw goat milk. These results are similar to those reported by Ryser and Marth (1987a) for inoculation of vat milk with *L.monocytogenes.*

The level of *L.monocytogenes* found in raw cow milk is usually very low at a detection level of 1 cfu/ml or less (Harvey and Gilmour 1992, Fenlon and Wilson 1989). Further studies were therefore carried out on the growth characteristics of *L.monocytogenes* inoculated at levels of 1 and 10 cfu/ml into milk prior to production of Camembert cheese. The results are shown in Figure 7. With a starting inoculum level of 1 cfu/ml, no *L.monocytogenes* could be detected directly after manufacture but *L.monocytogenes* was detected by direct plating after 7 days. With a starting inoculum level of 10 cfu/ml, *L.monocytogenes* increased by ten fold during manufacture but decreased within 7 days. During cheese ripening, *L.monocytogenes* at both inoculum levels continued to increase in numbers in a similar fashion and reached levels of 1.39×10^4 and 5.37×10^4 cfu/g, respectively within 35 days.

This type of cheese can be sold to the public after the *P.camemberti* mould has developed sufficiently to cover the cheese, which in this case was 14

days. The cheese can, however, be held at 4°C, 90% Relative Humidity (RH) to allow further ripening and can be held for up to 5-7 weeks after production before being sold.

The duration of ripening was continued for a further 14 days to show the level of survival of *L.monocytogenes* in over-ripe cheese (Figure 7). At the low inoculum level, *L.monocytogenes* decreased on storage while the higher inoculum level continued to rise. These results show that even at low levels of *L.monocytogenes* (1 cfu/ml) in raw milk, this organism will survive cheese manufacture and will continue to grow on storage at refrigeration temperatures (2-8°C) to reach high levels. This must be considered a potentially serious risk to public health.

In the dipping experiments, the results (Table 15) show that immediately after dipping the two week old Camembert cheese (i.e. surface completely covered with the *P.camemberti* white mould) in distilled water containing *L.monocytogenes* at a level of 10^3 cfu/ml, *L.monocytogenes* could not be detected by direct plating of the cheese from outside or inside of the cheese. The cheese used in this experiment was shown to be *Listeria* free before being dipped. Only after enrichment procedures could *L.monocytogenes* be isolated from the outside of the cheese after being dipped. The results further showed that as *L.monocytogenes* grows on the outside of the cheese, the organism must also migrate into the cheese as levels from inside the cheese continued to rise throughout the six weeks of sampling. The milk used to produce this cheese did not contain *L.monocytogenes* so the organism could have only been introduced into the cheese at the time of inoculation of the cheese since it was

shown to be free of *Listeria* after manufacture.

Similar results were shown using a higher surface contamination level of *L.monocytogenes* from 10^3 to 10^5 cfu/ml (Table 16). It should be noted that a slight inhibitory effect of *P.camemberti* strain Niege was detected (Table 12) which was most pronounced within the first week after contamination. After this time little difference in the growth rate of *L.monocytogenes* was shown between the two *P.camemberti* strains.

5.4 DISCUSSION

The results from the blue-veined cheese experiments (Table 14) made from raw milk shows that this cheese does not appear to present a serious risk of causing listeriosis, since *L.monocytogenes* did not survive the long-term storage (up to 5 months) required for the ripening of this cheese. The strain of mould used in cheese manufacture did not appear to alter the survival of *L.monocytogenes* in the cheese (Table 14). However, this was difficult to show due to the lack of growth and recovery of *L.monocytogenes* after initial production of the cheese as recovery after 4 weeks and until the end of ripening was by enrichment only. Actual cfu/ml of *L.monocytogenes* after enrichment were not carried out but only the presence or absence by the streak plate method. There was no effect on growth of *L.monocytogenes* by fungal strains used in production of this cheese.

The intensity of the biochemical activity of *P.roqueforti* varies considerably among strains and the choice of strain has a major effect on the quality of blue-veined cheese. Proteolysis, lipolysis and resultant production of

free fatty acids due to *P.roqueforti* in blue-veined cheese are very high compared with other cheese varieties. Methyl ketones are formed via the fatty acid β-oxidation pathway (Law 1984). Salt content (in the range of 3-5%) and moisture (40-45%) are particularly high in blue-veined cheese and will result in a high brine concentration in the cheese. All these factors will produce an unfavourable environment for the growth of *L.monocytogenes.* These experiments highlight the inability of *L.monocytogenes* to survive during production and ripening of this type of cheese.

There were no significant differences in the growth of *L.monocytogenes* in Camembert cheese during ripening between the two milk types used and no effect due to pasteurisation before inoculation. The highest survival levels in Camembert cheese after ripening was attained in raw goat milk using *P.camemberti* strain IMI 129208. From the impedance study (see Chapter 4), *P.camemberti* strain NIEGE had a significant inhibitory effect on the growth of *L.monocytogenes* while *P.camemberti* strains IMI 129208 and SAM 2 had no significant effect on the growth of *L.monocytogenes.* Survival and growth of *L.monocytogenes* was however unaltered by the *P.camemberti* strain used in the production of Camembert cheese (Figures 4-7). However, in the surface contamination experiments (Tables 15 and 16) it seems that the highest levels of *L.monocytogenes* were shown with *P.camemberti* strain SAM 2 and lowest levels with Niege. This indicates that *P.camemberti* strain Niege was inhibiting *L.monocytogenes* slightly since levels were roughly 0.5 $\log_{10}$ lower than with SAM 2. This correlates with the findings from the impedance study (see

Chapter 4) that strain Niege decreased the growth of *L.monocytogenes* compared to other *P.camemberti* strains including SAM 2. It is interesting that this inhibitory effect was detectable in experiments where *L.monocytogenes* was applied to the cheese in a manner which brought it directly in contact with the mature mould growth on the cheese surface. This initimate contact between the two organisms and the well established fungal growth may account for detectable levels of inhibition of *L.monocytogenes.*

The European Community Council Directive (92/46/EC) states that the microbial standard for *L.monocytogenes* in milk-based products on removal from the processing establishment is that it must be absent in 25g or 25ml of cheese (other than hard cheese) and 1g or 1ml of other products (Anon 1992) . This standard includes all soft-cheese. It is essential therefore that this organism should be absent in this type of cheese.

These results therefore suggest that Camembert cheese should only be manufactured using milk that has been determined not to be contaminated by *L.monocytogenes* as experiments in this study have shown that even with only 1 cfu/ml *L.monocytogenes* in milk, it is enough to produce a cheese with high levels of *L.monocytogenes* in the final product (Figure 7).

The results in this chapter also suggest that post-manufacture contamination of Camembert cheese with *L.monocytogenes* can result in a persistently infected cheese that could represent a health hazard if consumed by members of the public. Post-manufacture contamination of blue-veined cheese with *L.monocytogenes* was not studied in this work, but from a recent report showing high levels of *L.monocytogenes* in a blue-veined cheese manufactured

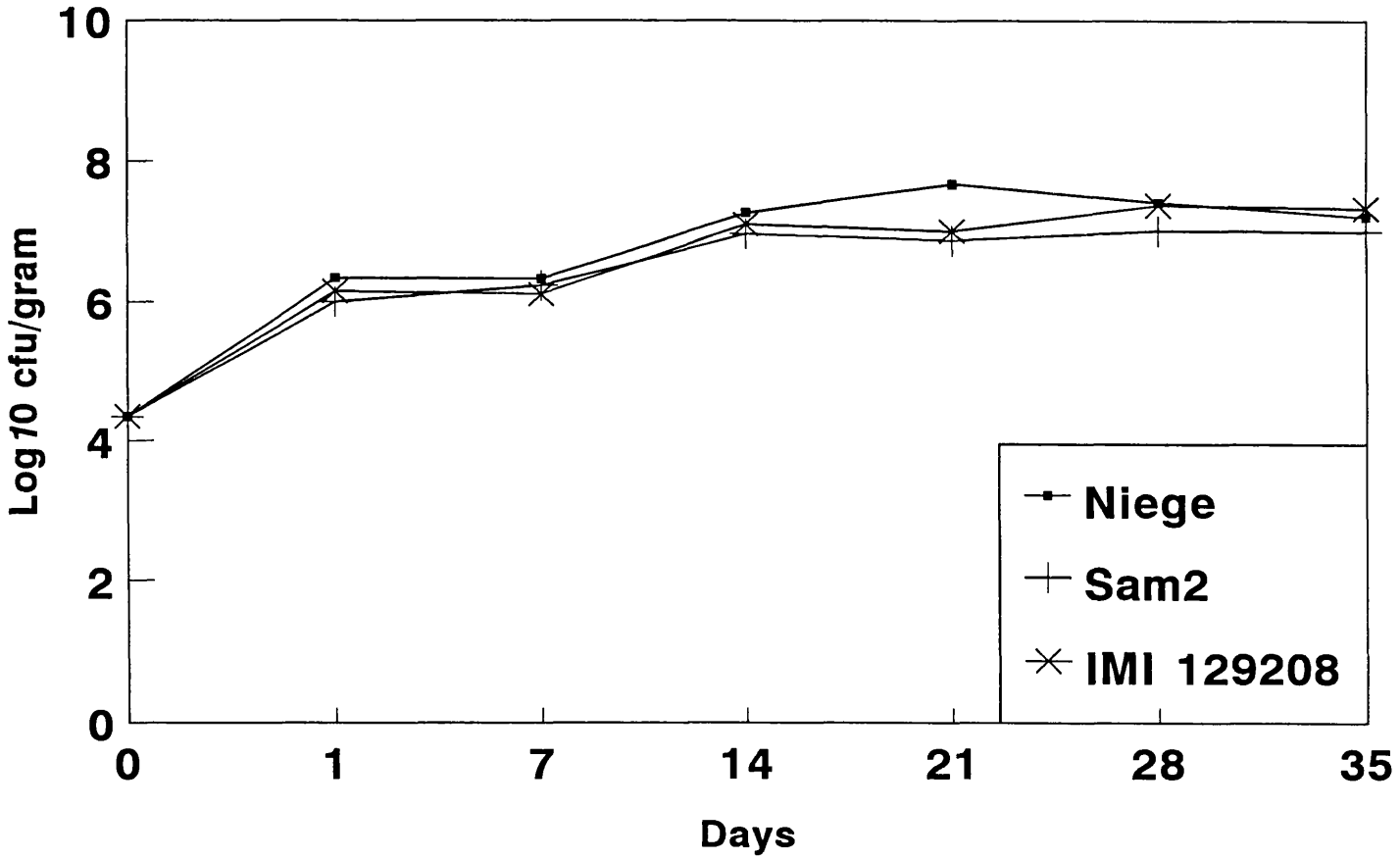
for raw sheep milk (manufactured by the same method outlined in this study), it would seem likely that post-manufacture contamination with *L.monocytogenes* could occur (Anon, 1995).

TABLE 14. Survival of *Listeria monocytogenes* (SITC 404/2) in Blue-veined cheese

Sample (weeks)	*Penicillium roqueforti* strain		
	IMI 148775	NO-N	PRB 6
	cfu/g or cfu/ml[1,2]		
0(whey)	<1	<1	<1
0(cheese)	$1.91x10^5$	$1.73x10^5$	$2.90x10^5$
4	+	+	+
8	+	+	+
12	-	-	-
16	-	-	-
20	-	-	-

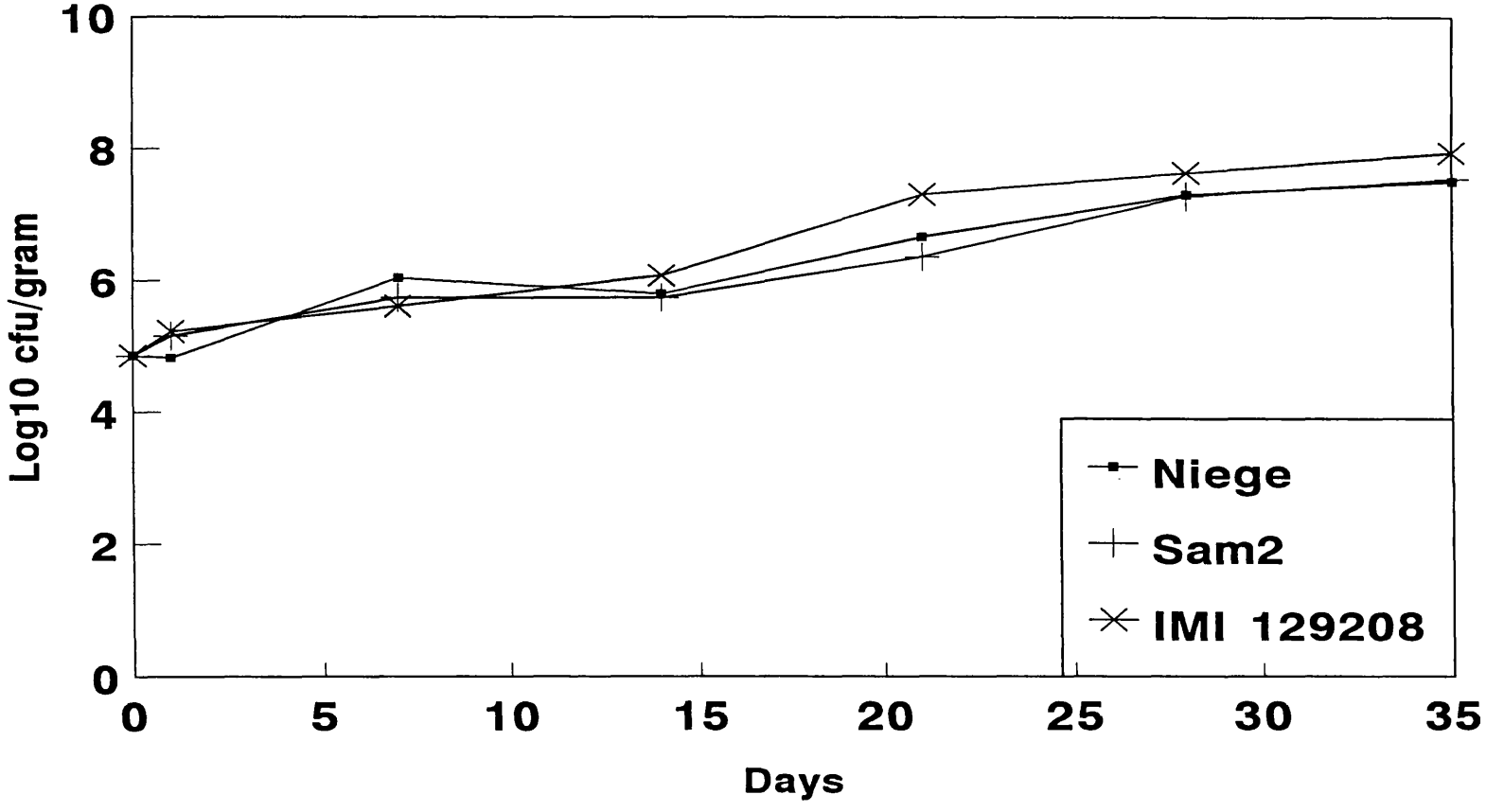
\+ *L.monocytogenes* detected after enrichment of 25g cheese.
\- No *L.monocytogenes* detected after enrichment of 25g cheese.
[1] Mean of three trials.
[2] Initial inoculum between $1.15x10^4$ - $5x10^4$ cfu/ml.
<1 No bacteria recovered, detection limit was 1 cfu/ml.

FIGURE 4. Survival of L.monocytogenes in Camembert cheese using different fungal strains with raw cow milk *



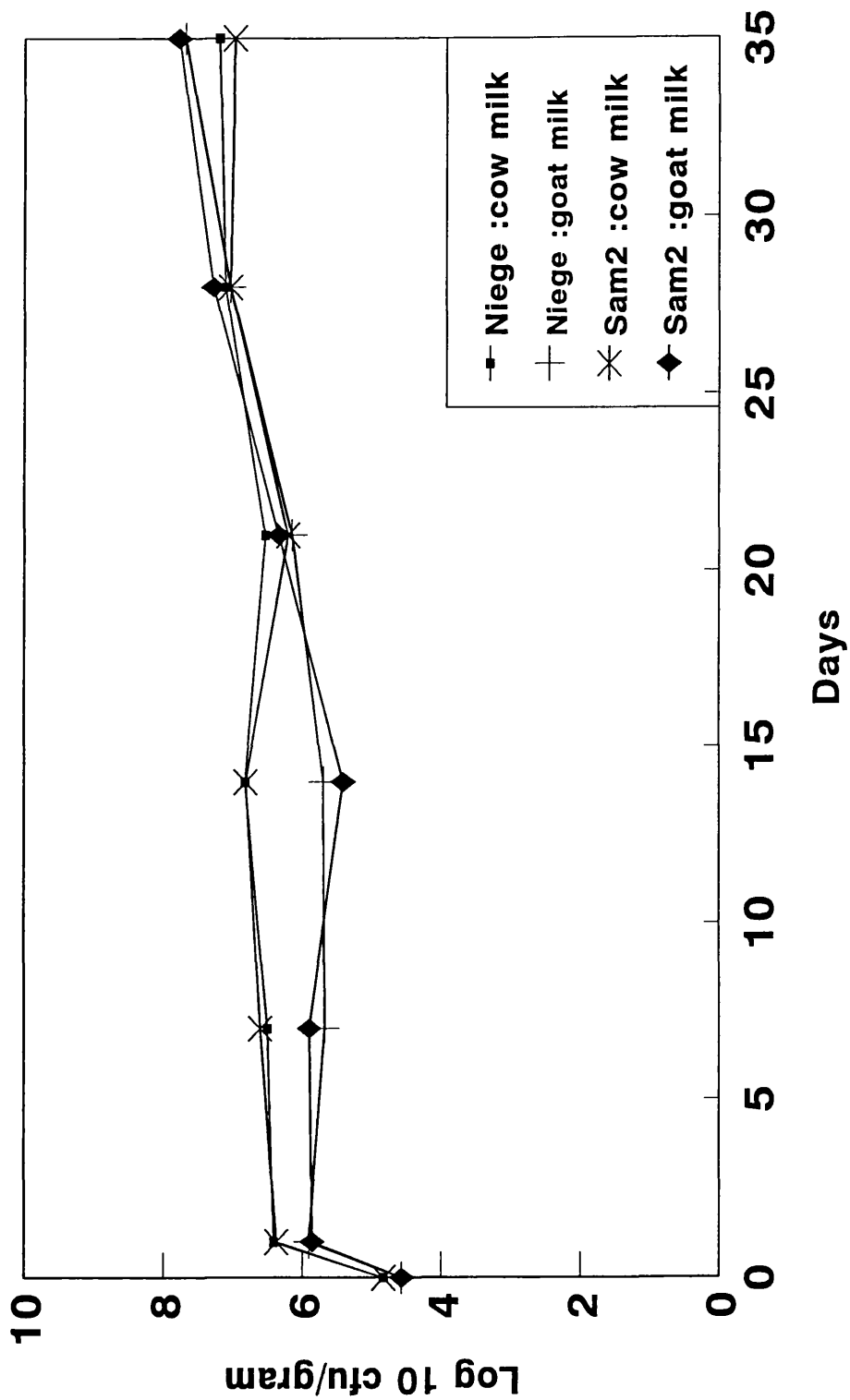
* Mean of two trials

FIGURE 5. Survival of L.monocytogenes in Camembert cheese using different fungal strains with raw goat milk *



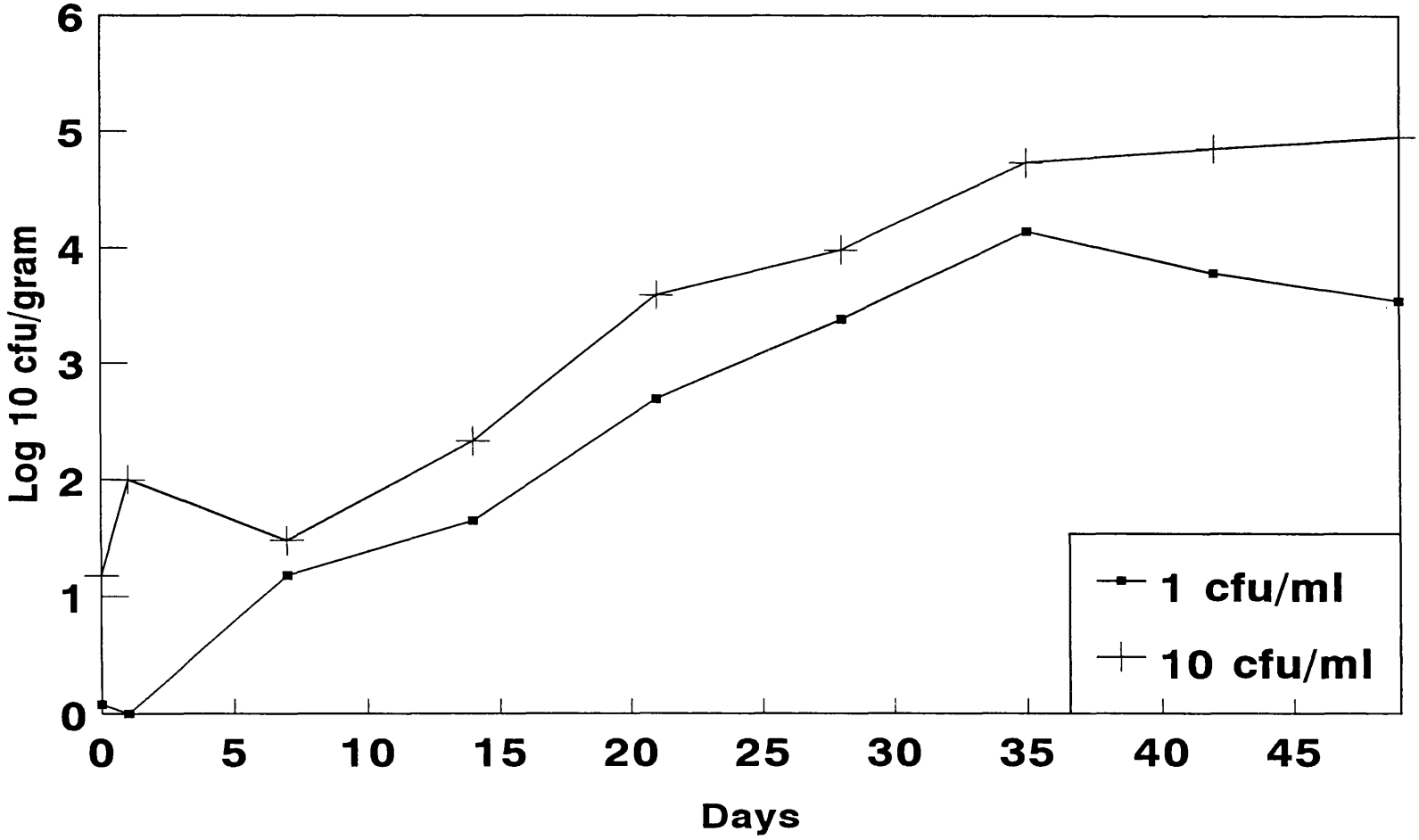
* Mean of two trials

FIGURE 6. Survival of L.monocytogenes in Camembert cheese using different fungal strains with pasteurised milks *



* Mean of two trials

FIGURE 7. Survival of L.monocytogenes at various levels of inoculum in Camembert cheese *



* Mean of two trials

TABLE 15. Survival of *Listeria monocytogenes* as a surface contaminant of Camembert cheese at a level of 10^3 cfu/ml

Weeks after inoculation	NIEGE		SAM 2	
	Outside	Inside	Outside	Inside
	CFU/G*			
0	0 (+)	0 (-)	0 (+)	0 (-)
1	65	0 (+)	$2.4x10^2$	0 (-)
2	$9.6x10^3$	10	$8.8x10^3$	5
3	$4.9x10^4$	10	$4.2x10^3$	15
4	$9.6x10^3$	20	$5.4x10^4$	20
5	$4.7x10^4$	35	$3.2x10^5$	25

(+) samples positive for *L.monocytogenes* after enrichment.
(-) samples negative for *L.monocytogenes* after enrichment at a detection limit of 1 cfu/ml.
* Mean of duplicate counts taken over 2 occasions.

TABLE 16. Survival of *Listeria monocytogenes* as a surface contaminant of Camembert cheese at a level of 10^5 cfu/ml

Weeks after inoculation	NIEGE		SAM 2	
	Outside	Inside	Outside	Inside
	CFU/G*			
0	$2.8x10^4$	$9.8x10^2$	$1.6x10^4$	$2.8x10^2$
1	$2.2x10^5$	40	$1.2x10^6$	$1.4x10^3$
2	$3.3x10^6$	80	$4.5x10^6$	$7.9x10^3$
3	$2.7x10^6$	$2.3x10^2$	$1.1x10^7$	$1.4x10^4$
4	$8.8x10^5$	$3.5x10^2$	$5.4x10^7$	$8.5x10^3$
5	$3.7x10^6$	$1.5x10^3$	$1.2x10^7$	$1.2x10^4$

* Mean of duplicate counts

CHAPTER 6

EFFECT OF HEAT TREATMENT ON *LISTERIA MONOCYTOGENES* AND GRAM-NEGATIVE BACTERIA IN SHEEP, COW AND GOAT MILKS

6.1 INTRODUCTION

The heat resistance of *L.monocytogenes* has been in question for a number of years. Bearns and Girard (1958), who used the "holding technique" of pasteurisation (61.7°C for 35 min), showed that viable *L. monocytogenes* could survive in cows milk whenever the initial number was 5 x 10^4 cfu/ml or greater. This was also confirmed by Donnelly *et al.* (1987) who used this method, but with another "sealed tube" method they also showed that with an inoculum of 10^6 - 10^7 cfu/ml, *L.monocytogenes* could not survive under the same conditions of heating. Several other workers have also demonstrated using different methods that *L. monocytogenes* cannot survive HTST pasteurisation (71.7°C 15 s) in cow milk (Bradshaw *et al.*, 1985; Beckers *et al.*, 1987; Lovett *et al.*, 1990).

These workers have published findings on the thermal resistance of *L.monocytogenes* with cow (bovine) whole or skimmed milk or in broths. No published work of this type has used sheep (ovine) or goat (caprine) milks.

Many types of cheese are produced from a variety of milk types or from a combination of two or more (e.g. Spanish Manchego cheese). All have varying levels of fat content, bacterial flora and heat treatments. Psychotrophic Gram negative bacilli are common contaminants of raw milk (Bramley and McKinnon, 1990) which can grow at low temperatures and cause spoilage. The best means of ensuring that pathogens such as *L.monocytogenes* and

important spoilage organisms are not transferred to cheese and other dairy products is to adequately pasteurise the milk. Sheep milk is generally of a higher fat content (5.8-9.1% weight) than cow (3.2-5.1% weight) or goat milk (2.8-6.5% weight) (Tamine *et al.*, 1991) and it is a concern that this higher fat content may protect bacteria from the effects of heating. To examine this possibility, this work investigated the survival of *L.monocytogenes, L.innocua* and Gram negative psychrotrophic spoilage bacteria in heat treated sheep, cow and goat milks with varying fat levels.

6.2 EXPERIMENTAL PROCEDURES

6.2.1 CULTURES

Three *L. monocytogenes* strains, SITC 12/1A, SITC 404/2 and NCTC 7973 and one *Listeria innocua* strain, SITC 236/2/8 (see section 2.1.1) were used in this study. Four psychrotrophic Gram-negative bacilli strains were also used which are all common milk isolates. These were *Pseudomonas fluorescens* NCDO 2085 , *Citrobacter freundii* GTE 022, *Acinetobacter lwoffi* GTE 024 and *Klebsiella ozoaenae* GTE 019 (see section 2.1.1). The *Listeria* strains were grown in TSB (see section 2.1.4) and all other strains were grown in NB at (see section 2.1.4) 37°C for 24 h to prepare inocula.

6.2.2 PREPARATION OF MILK SAMPLES

Raw cow and goat milks were obtained on site from the Hannah Research Institute and raw sheep milk was collected from a local sheep farm and maintained at between 2 - 8°C until arrival at the Institute. The milk was

dispensed into sterile containers (1 l) and centrifuged at 56g (Mistral 6L). The milk was separated from the top cream layer by suction and then filtered through Whatman No. 1, 15 cm filter paper to make skim milk.

The fat percentage of whole and skim milk was estimated using the Gerber method (Anon, 1989). The skim milk and separated fat were homogenised (Silverson Emulsifier, Chesham, Bucks) until the fat was dispersed in the milk to the required percentage at 0, 5 or 10% fat (w/v). In some experiments, where indicated, whole milk with the original fat content (without separation or resuspension) was used.

6.2.3. TEMPERATURE EXPERIMENTS

Water bath

Milk was dispensed in 10 ml volumes into duplicate sterile glass test tubes with lids. One hundred microlitres of 24 h cultures were added to each tube and mixed. The samples were placed in a water bath at 65 (±0.2), 68 (±0.2) or 72 (±0.2)^{o}C for 0, 15, 30 or 45 minutes. The samples took up to 2 min 15 sec (at 72^{o}C) to equilibriate at the required temperature and were cooled as quickly as possible after heating by immersion in an ice bath. All trials were repeated on three separate occasions.

Plate pasteuriser

An APV Junior heat exchanger system modified to heat samples up to 140^{o}C (Junior Paraflow, APV Baker, Derby) was used. Milk was inoculated with *Listeria* spp. to give 1 x 10^{6} cfu/ml. The milk was heated at 68, 70, 72 and 74^{o}C with a holding time of 15 s. The holding time was achieved by the

insertion of a tube of appropriate length in the holding section with a product flow of 90 l h^{-1}. Immediately after heating the milk samples were cooled as quickly as possible by immersion in an ice bath. All trials were repeated on two separate occasions.

Total plate counts were determined by spread plating (Stainer *et al.*, 1987) 2x500 µl samples (with appropriate dilutions in MRD) (see section 2.1.4) in duplicate at each heating interval on either MA for Gram-negative bacilli or on Oxford agar for *Listeria* spp. All plates were counted after incubation at 37°C for 48 h using a Plate Counter (Spiral Systems Inc) (see section 2.2.1) to calculate cfu/ml of bacteria recovered.

Five presumptive *Listeria* colonies were selected from Oxford agar after each heating interval for identification (see section 2.2.3). Representative colonies of Gram-negative bacilli were initially identified by Gram stain and the oxidase test. Oxidase positive isolates (i.e. *Pseudomonas fluorescens)* were further identified by Oxi/Ferm tubes (Roche) while oxidase negative isolates (i.e. *Acinetobacter lwoffi*, *Citrobacter freundii* and *Klebsiella ozoaenae*) were further identified with Enterotube II (Roche).

6.3 RESULTS

6.3.1 HEAT TREATMENT OF GRAM-NEGATIVE BACTERIA IN MILK

Trials were carried out by the water bath method using inocula of 10^6 - 10^7 cfu/ml of four strains of Gram-negative bacteria heated in cow, goat and sheep milks containing 0, 5 or 10% fat. Results showed that no organisms were recoverable within 15 min from all cow and goat milk samples and sheep

milk with 0% fat (results not shown), while for sheep milk samples containing 5 and 10% fat, heating for 30 min at 65°C was required before there were no organisms recoverable (Table 17). This indicated that sheep milk samples containing 5 or 10% fat had a protective effect on the survival of the organisms during heating.

The survival of Gram-negative bacteria was further studied in whole sheep milk (6.58% fat) using the water bath method and heating at 65, 68 and 72°C for 15 or 30 min. It was shown that even at 72°C 3 out of 4 of these bacterial isolates could survive for 15 min but none were recoverable after heating for 30 minutes (Table 18).

Further trials with skim cow and sheep milks made up to 10% fat content with either homologous or heterologous fat (Table 19) showed that sheep fat protected Gram-negative bacteria from heating at 65°C for 15 min when added to cow and sheep skim milks. However, cow fat added to cow skim milk did not protect the organism from heating, but conversely cow fat added to sheep skim milk was protective. The results show that both sheep skim milk and sheep milk fat have a protective factor present which increases the survival of these organisms during heating (Tables 17 and 19).

6.3.2 HEAT TREATMENT OF *LISTERIA* SPECIES IN MILK

Trials with inocula of 10^6 - 10^7 cfu/ml of four *Listeria* strains in cow and sheep milk containing 0, 5 or 10% fat were conducted using the water bath method at 65°C. Results showed that no organisms were recoverable within 45 min for all cow milk samples (Table 20) and skim sheep milk (no fat) (Table 21). Cow skim milk containing 10% fat did however show some protective

effect in that *Listeria* survived in this sample for up to 30 min. Sheep milk samples containing 5 and 10% fat still contained viable organisms after 45 min (Table 21).

Further trials with ten percent homologous or heterologous fat (Table 22) in skim cow and sheep milks showed sheep fat to be protective to all *Listeria* strains after heating at 65°C for 45 min in both cow and sheep skim milks. Cow fat added to cow skim milk, however did not protect these bacteria from heating after 30 min at 65°C, but cow fat added to sheep skim was protective, although to a lesser extent than sheep fat added to sheep milk.

The protective effects of sheep skim milk and sheep milk fat shown with Gram-negative bacteria (Tables 17 and 19) were therefore also observed with the *Listeria* spp. and strains used (Tables 20-22).

The survival of *L. monocytogenes* (SITC 12/1A) was further studied in sheep milk containing 10% fat. Using the water bath method, varying levels of *L.monocytogenes* (SITC 12/1A) inoculum were heated at 65°C for up to 45 min. A starting inoculum as low as 8.9×10^2 cfu/ml survived for 30 min but no organisms were recovered after heating for 45 min (Table 23).

The survival of *Listeria* spp. in sheep milk was further studied in whole sheep milk (7% fat) and the water bath method heated at 65, 68 and 72°C for 15, 30 or 45 min (Table 24). It was shown that at 68°C all bacterial strains could survive for 30 min but no organisms were recovered within 15 min at 72°C.

When whole sheep milk containing 1×10^6 cfu/ml of *L. monocytogenes* (SITC 12/1A) was heated in a plate pasteuriser at 68, 70, 72 and 74°C for 15 s

only 10 cfu/ml survived at 68°C and at all other temperatures no organisms were recovered. The same inoculum of *L. monocytogenes* did not survive in whole cow (3.27% fat) or goat (2.85%) milk when heated at 68,70,72 and 74°C for 15 s on the plate pasteuriser (results not shown).

6.4. DISCUSSION

Sheep milk was found to have factors which protected bacteria against heating. The protective effect seemed to be specific for sheep milk and not solely due to fat percentage since cow and goat milks artificially made up to 10% homologous fat content were not found to protect Gram-negative bacteria against heating. Cow's milk artificially made up to 10% homologous fat content also did not protect strains of *Listeria monocytogenes* or *Listeria innocua* against heating to the same extent as 5 or 10% sheep fat.

Listeria innocua was used in this study to determine whether there were any differences in the susceptibility to heat treatments between non-pathogenic *L.innocua* and pathogenic *L.monocytogenes.* Three different *L.monocytogenes* isolates were studied to determine whether there were differences in the susceptibility of these isolates due to the heat treatments. The results show that all the heat treatments gave similar results for the survival of all the *Listeria* species and strains examined.

The Milk (Special Designation) Regulations 1989 state that for pasteurised whole cow milk the heat treatment should be not less than 62.8°C and not more than 65.6°C for at least 30 min (batch pasteurisation) or not less than 71.7°C for at least 15 s (plate pasteurisation) or an equivalent process.

The results for the heating of sheep milk showed that even a small level of inoculum of *L.monocytogenes* (i.e. 8.9 x 10^2 cfu/ml) could survive the batch pasteurisation conditions specified in the above regulation. The results obtained using the plate pasteuriser indicate that it was far more efficient at heat killing than the water bath method.

Bradshaw *et al*. (1985) stated that *L.monocytogenes* strain Scott A in raw milk had a D-value at 68.9°C of 3 s and at 71.7°C of 0.09 s. The results obtained here using the plate pasteuriser at 72°C were in agreement with these authors in that *L.monocytogenes* did not survive pasteurisation at 72°C for 15 s, but their findings suggest *L.monocytogenes* would have survived at 68°C in whole goat, cow and sheep milks. The findings reported here showed that *L. monocytogenes* was not detectable after heating at 68°C in cow and goat milks but survival did occur in sheep milk. This again indicated that *L.monocytogenes* (SITC 12/1A) has a greater heat resistance in whole sheep milk compared to whole cow or goat milk.

The Gram-negative bacteria used in this study were less heat resistant at 65°C in whole cow and sheep milk than the four *Listeria* strains used (three *L. monocytogenes* and one *L. innocua*) but could survive heating at 72°C for 15 min in the water bath method while the four *Listeria* strains could not. The reason for this finding is unclear as previous work has indicated *Listeria* is generally more heat resistant than other bacteria (Mackey and Bratchell, 1989).

Protective factor(s) present in sheep skim milk and sheep milk fat were demonstrated with both Gram-negative and Gram-positive bacteria.

Since psychrotrophic Gram-negative organisms can survive heating at 72°C for 15 min and *Listeria* spp. survive heating at 65°C for 45 min it would therefore be advisable to pasteurise whole sheep milk at 72°C for 30 min with the water bath method. Whole sheep, cow and goat milks containing even high levels of *L.monocytogenes* (1×10^6 cfu/ml) could not survive the current HTST plate pasteurisation protocol.

TABLE 17. Survival of Gram-negative bacterial strains at 65 °C for various times as a function of fat content of sheep milk

Bacterial strain	Colony forming units per/ml after (minutes) 0	15	30	45
	5% fat added to skim			
Pseudomonas fluorescens NCDO 2085	$>5x10^7$	$8.5x10^3$	<1	<1
Citrobacter freundii GTE 022	$2.8x10^7$	$1.8x10^3$	<1	<1
Acinetobacter lwoffi GTE 024	$2.1x10^7$	$5.7x10^3$	<1	<1
Klebsiella ozoaenae GTE 019	$2.0x10^7$	$7.6x10^2$	<1	<1
	10% fat added to skim			
Pseudomonas fluorescens NCDO 2085	$9.5x10^6$	$2.2x10^4$	<1	<1
Citrobacter freundii GTE 022	$1.1x10^7$	$9.1x10^2$	<1	<1
Acinetobacter lwoffi GTE 024	$1.0x10^7$	$5.7x10^3$	<1	<1
Klebsiella ozoaenae GTE 019	$1.0x10^7$	$4.2x10^3$	<1	<1

<1 = No bacteria recovered, detection limit was 1 cfu/ml

TABLE 18. Survival of Gram-negative bacterial strains at 65, 68 and 72 °C for various times in whole sheep milk (6.58% fat)

°C	Bacterial strain	Colony forming units/ml after (minutes) 0	15	30
65	*Pseudomonas fluorescens* NCDO 2085	7.0×10^6	6.4×10^3	< 1
	Citrobacter freundii GTE 022	8.6×10^6	8.1×10^3	< 1
	Acinetobacter lwoffi GTE 024	6.8×10^6	1.0×10^3	< 1
	Klebsiella ozoaenae GTE 019	3.4×10^6	9.2×10^3	< 1
68	*Pseudomonas fluorescens* NCDO 2085	7.0×10^6	2.6×10^3	< 1
	Citrobacter freundii GTE 022	8.6×10^6	5.0×10^2	< 1
	Acinetobacter lwoffi GTE 024	6.8×10^6	3.8×10^2	< 1
	Klebsiella ozoaenae GTE 019	3.4×10^6	4.9×10^2	< 1
72	*Pseudomonas fluorescens* NCDO 2085	7.0×10^6	1.9×10^3	< 1
	Citrobacter freundii GTE 022	8.6×10^6	1.9×10^2	< 1
	Acinetobacter lwoffi GTE 024	6.8×10^6	2.6×10^2	< 1
	Klebsiella ozoaenae GTE 019	3.4×10^6	< 1	< 1

< 1 = No bacteria recovered, detection limit was 1 cfu/ml

TABLE 19. Survival of Gram-negative bacteria at 65 °C for 15 minutes in sheep or cow skim milk supplemented with sheep or cow milk fat

Bacterial strain	Colony forming units/ml after (minutes) 0	15
	Sheep skimmed milk + 10% sheep fat	
Pseudomonas fluorescens NCDO 2085	7.3×10^6	5.5×10^3
Citrobacter freundii GTE 022	9.3×10^6	8.8×10^3
Acinetobacter lwoffi GTE 024	5.0×10^6	6.8×10^3
Klebsiella ozoaenae GTE 019	3.6×10^6	8.6×10^3
	Sheep skimmed milk + 10% cow fat	
Pseudomonas fluorescens NCDO 2085	1.9×10^6	1.7×10^4
Citrobacter freundii GTE 022	5.5×10^6	1.3×10^4
Acinetobacter lwoffi GTE 024	2.3×10^6	7.8×10^3
Klebsiella ozoaenae GTE 019	3.1×10^6	1.0×10^4
	Cow skimmed milk + 10% cow fat	
Pseudomonas fluorescens NCDO 2085	1.6×10^7	< 1
Citrobacter freundii GTE 022	9.3×10^6	< 1
Acinetobacter lwoffi GTE 024	2.5×10^6	< 1
Klebsiella ozoaenae GTE 019	4.6×10^6	< 1
	Cow skimmed milk + 10% sheep fat	
Pseudomonas fluorescens NCDO 2085	1.1×10^7	4.9×10^3
Citrobacter freundii GTE 022	9.8×10^6	1.5×10^3
Acinetobacter lwoffi GTE 024	5.4×10^6	6.5×10^3
Klebsiella ozoaenae GTE 019	4.5×10^6	1.5×10^4

< 1 = No bacteria recovered, detection limit was 1 cfu/ml

TABLE 20. Survival of *Listeria* strains at 65°C for various times as a function of fat content of cow milk

Bacterial strain	Colony forming units/ml after (minutes)* 0	15	30	45
	No fat added to skim			
L. monocytogenes SITC 12/1A	3.00×10^6	< 1	< 1	< 1
L. monocytogenes SITC 404/2	2.80×10^6	< 1	< 1	< 1
L. monocytogenes NCTC 7973	6.15×10^6	1.80×10^2	< 1	< 1
L. innocua SITC 236/2/8	6.60×10^6	85	< 1	< 1
	5% fat added to skim			
L. monocytogenes SITC 12/1A	3.37×10^6	55	< 1	< 1
L. monocytogenes SITC 404/2	2.17×10^6	1.50×10^2	< 1	< 1
L. monocytogenes NCTC 7973	6.40×10^6	5.35×10^2	< 1	< 1
L. innocua SITC 236/2/8	3.82×10^6	2.43×10^2	< 1	< 1
	10% fat added to skim			
L. monocytogenes SITC 12/1A	2.38×10^6	4.53×10^3	3.68×10^2	< 1
L. monocytogenes SITC 404/2	4.76×10^6	3.83×10^3	14	< 1
L. monocytogenes NCTC 7973	5.18×10^6	1.57×10^3	55	< 1
L. innocua SITC 236/2/8	8.14×10^6	8.14×10^3	4.12×10^2	< 1

*Mean of 12 replicate counts taken over 3 occasions.
< 1 = No bacteria recovered, detection limit was 1 cfu/ml.

TABLE 21. Survival of *Listeria* strains at 65°C for various times as a function of fat content of sheep milk

Bacterial strain	Colony forming units/ml after (minutes)* 0	15	30	45
		No fat added to skim		
L. monocytogenes SITC 12/1A	7.93×10^6	6.92×10^3	62.5	< 1
L. monocytogenes SITC 404/2	6.55×10^6	3.18×10^3	14.0	< 1
L. monocytogenes NCTC 7973	1.59×10^7	1.61×10^4	4.51×10^2	< 1
L. innocua SITC 236/2/8	1.61×10^7	4.28×10^4	97.0	< 1
		5% fat added to skim		
L. monocytogenes SITC 12/1A	1.01×10^7	1.05×10^4	4.22×10^3	2.13×10^3
L. monocytogenes SITC 404/2	7.90×10^6	2.44×10^3	4.09×10^2	28
L. monocytogenes NCTC 7973	1.32×10^7	2.89×10^3	2.53×10^3	< 1
L. innocua SITC 236/2/8	1.63×10^7	1.83×10^4	2.72×10^3	2.01×10^2
		10% fat added to skim		
L. monocytogenes SITC 12/1A	1.12×10^7	6.29×10^3	4.58×10^3	1.65×10^3
L. monocytogenes SITC 404/2	6.83×10^6	6.75×10^3	8.16×10^2	4.99×10^2
L. monocytogenes NCTC 7973	5.16×10^6	1.10×10^3	1.32×10^2	77
L. innocua SITC 236/2/8	1.87×10^7	6.04×10^3	3.43×10^3	9.79×10^2

*Mean of 12 replicate counts taken over 3 occasions.
< 1 = No bacteria recovered, detection limit was 1 cfu/ml.

TABLE 22. Survival of *Listeria* strains at 65°C for various times in sheep or cow skim milk supplemented with sheep or cow fat

Bacterial strain	Colony forming units/ml after (minutes)* 0	15	30	45
	10% sheep fat added to sheep skim milk			
L. monocytogenes SITC 12/1A	2.25×10^{7}	1.93×10^{3}	1.32×10^{3}	2.50×10^{2}
L. monocytogenes SITC 404/2	1.08×10^{7}	2.45×10^{3}	1.01×10^{3}	6.50×10^{2}
L. monocytogenes NCTC 7973	1.50×10^{7}	2.77×10^{3}	9.50×10^{3}	3.11×10^{2}
L. innocua SITC 236/2/8	1.93×10^{7}	5.12×10^{3}	2.65×10^{3}	4.55×10^{2}
	10% cow fat added to sheep skim milk			
L. monocytogenes SITC 12/1A	2.25×10^{7}	1.53×10^{3}	1.85×10^{2}	20
L. monocytogenes SITC 404/2	2.49×10^{7}	1.37×10^{3}	4.22×10^{2}	95
L. monocytogenes NCTC 7973	2.18×10^{7}	3.11×10^{3}	3.34×10^{2}	65
L. innocua SITC 236/2/8	2.11×10^{7}	2.50×10^{3}	4.75×10^{2}	75
	10% cow fat added to cow skim milk			
L. monocytogenes SITC 12/1A	3.25×10^{7}	1.75×10^{3}	1.55×10^{2}	<1
L. monocytogenes SITC 404/2	1.58×10^{7}	1.20×10^{3}	2.12×10^{2}	<1
L. monocytogenes NCTC 7973	5.25×10^{7}	3.16×10^{3}	59	<1
L. innocua SITC 236/2/8	4.50×10^{7}	3.74×10^{3}	$1.7\ 3\times10^{2}$	<1
	10% sheep fat added to cow skim milk			
L. monocytogenes SITC 12/1A	2.08×10^{7}	7.66×10^{2}	3.00×10^{2}	1.40×10^{2}
L. monocytogenes SITC 404/2	1.26×10^{7}	3.75×10^{3}	4.78×10^{2}	1.94×10^{2}
L. monocytogenes NCTC 7973	1.75×10^{7}	3.02×10^{3}	2.25×10^{2}	85
L. innocua SITC 236/2/8	2.75×10^{7}	4.50×10^{3}	5.72×10^{2}	2.11×10^{2}

*Mean of 12 replicates taken on 3 occasions.
<1 = No bacteria recovered, detection limit was 1 cfu/ml.

TABLE 23. Survival of *Listeria monocytogenes* (SITC 12/1A) at 65°C for various times in sheep milk with 10% fat content

Start inoculum level (cfu/ml)	Colony forming units/ml after (minutes)*			
	0	15	30	45
neat	4.44×10^6	2.64×10^4	3.67×10^3	1.04×10^3
1/5 D	2.70×10^6	1.54×10^4	9.03×10^2	6.46×10^2
1/10 D	1.63×10^6	1.25×10^4	1.72×10^3	8.47×10^2
1/50 D	5.70×10^4	2.31×10^4	2.78×10^2	6.39×10^2
1/100 D	6.83×10^3	7.99×10^2	7.70×10^2	4.17×10^2
1/500 D	1.50×10^3	4.86×10^2	4.51×10^2	4.33×10^2
1/1000 D	1.00×10^3	2.01×10^2	1.18×10^2	8.33×10^1
1/5000 D	8.90×10^2	14	7	< 1

D = dilution using maximum recovery diluent (MRD).
*Mean of 8 replicates taken over 2 occasions.
< 1 = No bacteria recovered, detection limit was 1 cfu/ml.

TABLE 24. Numbers of *Listeria* spp. surviving heat treatment at 65, 68 and 72°C for various times in whole sheep milk (7% fat)

°C	Bacterial strain	Colony forming units/ml after (minutes)* 0	15	30	45
65	*L.monocytogenes* SITC 12/1A	2.48×10^7	2.13×10^4	3.00×10^3	1.60×10^2
	L.monocytogenes SITC 404/2	2.65×10^7	2.00×10^4	6.70×10^2	1.50×10^2
	L.monocytogenes NCTC 7973	2.09×10^7	9.25×10^3	4.20×10^2	1.35×10^2
	L.innocua SITC 236/2/8	3.25×10^7	6.00×10^4	3.00×10^3	5.00×10^2
68	*L.monocytogenes* SITC 12/1A	2.69×10^7	2.00×10^3	1.80×10^2	< 1
	L.monocytogenes SITC 404/2	2.23×10^7	1.72×10^3	5.40×10^2	< 1
	L.monocytogenes NCTC 7973	2.94×10^7	1.00×10^3	1.80×10^2	< 1
	L.innocua SITC 236/2/8	2.51×10^7	1.50×10^3	2.40×10^2	< 1
72	*L.monocytogenes* SITC 12/1A	2.76×10^7	< 1	< 1	< 1
	L.monocytogenes SITC 404/2	2.14×10^7	< 1	< 1	< 1
	L.monocytogenes NCTC 7973	3.06×10^7	< 1	< 1	< 1
	L. innocua SITC 236/2/8	2.97×10^7	< 1	< 1	< 1

*Mean of 12 replicates taken over 3 occasions.
< 1 = No bacteria recovered, detection limit was 1 cfu/ml.

CHAPTER 7

SURVIVAL OF *LISTERIA MONOCYTOGENES* IN THERMISED MILK AND SUBSEQUENT CHEESE MANUFACTURE

7.1 INTRODUCTION

In continental Europe, the process of thermisation is used to treat raw milk in order to extend cold-storage of the milk. Thermisation is not a replacement for pasteurisation (Gilmour *et al.*, 1981), but is intended to reduce psychrotrophic bacterial flora causing spoilage without causing considerable change to the properties of the raw milk. The International Dairy Federation (Anon, 1981) describes thermisation as "a system of heat treatment. It is carried out under continuous flow conditions and consists of heating to 63-65°C, holding for 15-20 s and then cooling, with the result that psychrotrophic bacteria are almost completely destroyed, while for the greater part the milk enzymes are unaffected". In Canada a treatment at 62.8°C or 65.6°C for 16 s is used for thermisation of milk intended for long-hold Canadian cheddar manufacture.

Thermisation does not have the same capabilities as pasteurisation for destroying pathogens. Mackay and Bratchell (1989) have shown that *L.monocytogenes* would survive in raw milk thermised at between 60 and 65°C for 16 s. Since *L.monocytogenes* is psychrotrophic in nature, surviving organisms are likely to grow on subsequent cold-storage.

This study was designed to determine whether and to what levels *L.monocytogenes* would survive thermisation treatments using a plate pasteuriser,

when starting levels were at approximately 10^3 cfu/ml. This level represents the worst possible scenario for raw milk considering that a recent examination of fresh dairy silo raw milk in Scotland showed *L.monocytogenes* to be present in 7.8% of silo milks with the highest count recovered being < 1.0 cfu/ml (see Chapter 3). Although *L.monocytogenes* can grow at 4°C, it has a lag time of about 36 h and a generation time of at least 24 h (Donnelly and Briggs, 1986) with one report suggesting 5-10 day (Rosenow and Marth, 1987).

This study was also designed to compare the recovery of "stressed" *L.monocytogenes* using two different *Listeria* enrichment broths after different heat treatments and cheese manufacture.

The study then aimed to determine the fate of *L.monocytogenes* in milk subjected to thermisation and then used to manufacture cheese and also further cold-storage and subsequent pasteurisation. In each case the fate of *L.monocytogenes* in thermised milk was compared with that in control experiments using raw milk.

The ability of thermisation to induce heat shock proteins in *L.monocytogenes* was also studied and findings related to whether induction of heat shock proteins would compromise subsequent pasteurisation as a means of killing *L.monocytogenes.*

7.2 EXPERIMENTAL PROCEDURES

7.2.1 CULTURES

Listeria monocytogenes strain Scott A (see section 2 .1.1) was used in all these experiments. *Penicillium roqueforti* strain Blue 18 and *P.candidium* S11 (see section 2.1.1) were used in the manufacture of Danish blue cheese while *Penicillium camemberti* strain Sam 2 (see Section 2.1.1) was used in the

manufacture of Camembert cheese. The starter culture used in all these experiments was MA 011 (see section 2.1.1).

7.2.2 MANUFACTURE OF CHEESE

The long-hold Canadian cheddar cheese was produced by the method outlined by Scott (1986) using cow milk. The Camembert cheese was produced by the method outlined by Scott (1986) for traditional Camembert soft cheese (Modern Technology) using cow milk. *P.camemberti* used was added to the milk (10 litres) in the cheese vat (see section 2.1.1). The Danish blue cheese was produced by the method outlined by Nielson and Ullum (1989) using sheep milk. *P.roqueforti* and *P.candidum* used were added to the milk (10 litres) in the cheese vat (see section 2.1.1).

7.2.3 PREPARATION OF MILK SAMPLES FOR CHEESE MANUFACTURE

A 24 h TSB (see section 2.1.4) culture of *L.monocytogenes* was inoculated into milk intended for cheese manufacture to give approximately 10^3 cfu/ml on three seperate occasions. After allowing the *Listeria* inoculum to ''adapt'' to the storage temperature of 4°C for 72 h, volumes of milk were thermised at 62.8 or 65.6°C for 16 s. An APV Junior heat exchanger system modified to heat samples to 140°C (Junior Paraflow, APV Baker, Derby) was used to thermise milk. It was set at 62.8 or 65.6°C with a holding time of 16 s. This was maintained by the insertion of a tube of appropriate length in the

holding section with a production flow of 90 l/h. The remaining volume of milk was given no heat treatment (control).

7.2.4 ENUMERATION OF *L.MONOCYTOGENES* IN CHEESE

Appropriate dilutions of raw milk, inoculated milk, inoculated milk after storage, after thermisation, during cheese manufacture and maturation were analysed to enumerate *L.monocytogenes* by direct plating of duplicate 2x500µl volumes spread plated (Stainer *et al.*, 1987) onto Oxford agar (see section 2.1.4) and also enrichment broth culture by the IDF method in LEB or by a modified enrichment in LRB (see section 2.1.4). The LRB had been shown to recover ''stressed'' *Listeria* more readily than LEB (Busch and Donnelly, 1991) and was included in these experiments to ensure optimised recovery of *L.monocytogenes.* Samples for bacteriological analysis were taken from the above milks, 24 hr cheese curd and a wedge was aseptically removed from the Canadian cheddar cheese at 2 weeks and thereafter during maturation monthly up until 9 months, from the Camembert cheese at 1 week and thereafter fortnightly up until 7 weeks and the Danish blue cheese at 1 week and thereafter at 2-3 week intervals up until 11 weeks. 25ml of milk and 25g cheese samples were added to 225 ml of LEB and LRB (see section 2.1.4) warmed to 45°C contained in sterile stomacher bag and blended for 2 min in a stomacher (Stomacher 400). Duplicate 2x500µl volumes of the enrichment broths and/or dilutions were directly plated onto the surface of Oxford agar so that well isolated colonies were obtained if present. The enrichment broths were then incubated at 30°C for 48 h and only plated if recovery of *L.monocytogenes* by direct plating was unsuccessful. The plates were incubated

at 37°C for 48 h. Colonies typical of those formed by *L.monocytogenes* were counted, and selected colonies were confirmed as *L.monocytogenes* according to the International Dairy Federation Procedure 143 (Anon, 1990) (see section 2.2.3).

7.2.5 THERMISATION AND SUBSEQUENT PASTEURISATION ON *L.MONOCYTOGENES* IN MILK

A series of studies were initiated to determine the fate of *L.monocytogenes* in thermised stored milk which was subsequently pasteurised (Figure 8). In the first three experiments (A-C) milk was inoculated with approximately 10^3 cfu/ml *L.monocytogenes* and held at 4°C for 24 h. Volumes of the milk were then thermised at 65.5°C for either 0, 15, 60 or 120 s and stored for either 1 or 2 days at 4°C before High Temperature Short Time (HTST) pasteurisation at 72°C for 15 s. Milk from each treatment was then further stored at 4°C for 1, 2 or 3 days. In the next three experiments milk was inoculated with approximately 10^6 cfu/ml with two being thermised at 65.6°C (D-E) and one at 62.8°C (F) and treated as before. Enumeration of *L.monocytogenes* was as before (see section 7.2.4).

7.2.6 INDUCTION OF HEAT RESISTANCE IN *L.MONOCYTOGENES*

A 24 h TSB (see section 2.1.4) culture of *L.monocytogenes* with approximately 10^8 cfu/ml was heat treated at 48°C for 15 or 30 minutes or given no pre-heat treatment (control). These samples were then subsequently heat treated at either 60 or 65°C for 5, 10 , 15, 20, 25 and 30 minutes. Enumeration of *L.monocytogenes* was as before (see section 7.2.4).

7.3 RESULTS

7.3.1 CANADIAN CHEDDAR CHEESE MADE FROM THERMISED MILK

Results for *L.monocytogenes* survival in long-hold Canadian cheddar cheese are shown in Tables 25-27. The inoculum ranged from $4.5x10^2$-$5.0x10^3$ cfu/ml in the milk. Little , if any growth occurred over the 3 day storage period at 4°C before thermisation of the milk as counts ranged from $5.57x10^2$-$9.0x10^3$ cfu/ml. *L.monocytogenes* was recovered by direct plating from 2 out of 3 batches of milk thermised at 62.8°C, but was recovered from all the batches by enrichment broth culture. The LRB gave higher numbers of *L.monocytogenes* than LEB. At 65.6°C, none of the 3 milk batches had *L.monocytogenes* recoverable by direct plating. However, 2 of these batches had *L.monocytogenes* recoverable after enrichment broth culture.

L.monocytogenes in raw milk made into Canadian long-hold cheddar cheese survived readily in the cheese curd and multiplied by 1-2 logs within 24 h, after which high numbers survived over the storage period of up to 9 months. In cheddar cheese made from milk thermised at 62.8°C, *L.monocytogenes* was recoverable in all 3 batches at least after enrichment broth culture. In batch 1 cheese (Table 25), *L.monocytogenes* was never recovered by direct plating but was recovered for up to 3 months by enrichment culture. In batch 2 and 3 (Tables 26 and 27), *L.monocytogenes* was isolated by direct plating for up to 8 months and by enrichment culture in batch 2 cheese at 9 months. In batch 2 and 3 , *L.monocytogenes* multiplied detectably in the first 24 h. Isolation counts from cheese reflected the counts initially recovered from the milk directly after thermisation. In cheddar cheese made from milk

thermised at 65.6^{0}C, *L.monocytogenes* was recovered at least from one point from each cheese after manufacture. Only in one cheese was *L.monocytogenes* isolated by direct plating for up to 2 weeks; thereafter, *L.monocytogenes* was isolated from this cheese batch for up to 7 months by enrichment culture (Table 26). In the other 2 batches (Table 25 and 27), *L.monocytogenes* was never isolated by direct plating and was isolated by enrichment culture for up to 2 months only. These counts again reflect the numbers of *L.monocytogenes* surviving in each milk batch after thermisation at 65.6^{o}C.

In milk batches 1-3 it was apparent that a thermisation temperature of 65.6^{o}C was more efficient at reducing *L.monocytogenes* counts in milk than was a temperature of 62.8^{o}C. Neither of these protocols were sufficient to ensure complete eradication of *L.monocytogenes* from cheddar cheese. Incidences of recovery and counts were however, generally lower than cheeses made from raw milk.

7.3.2 CAMEMBERT CHEESE MADE FROM THERMISED MILK

Results for survival of *L.monocytogenes* in Camembert cheese are shown in Table 28-30. The counts of *L.monocytogenes* in milk batches 4-6 were between 4.1x10^3-5.8x10^3 cfu/ml and between 4.4x10^3-6.8x10^3 cfu/ml after storage for 3 days at 4^{o}C. In Camembert cheese made from raw milk, *L.monocytogenes* was recoverable by direct plating right up to 7 weeks of maturation. The *L.monocytogenes* inoculum continued to multiply up to 10^6 cfu/g which is in agreement with previous findings reported in this study (see Chapter 5).

In Camembert cheese made from milk thermised at 62.8^{o}C (Tables

28-30), resultant counts of *L.monocytogenes* were lower than those obtained from raw milk cheese but surviving organisms grew and were detectable by direct plating at about 10^3 cfu/g up to 7 weeks maturation in each of the 3 batches of cheese made.

In Camembert cheese made from milk thermised at 65.6°C (Tables 28-30), results were variable from batch to batch. In milk batch 5, the Camembert cheese had counts of *L.monocytogenes* detectable by direct plating up to 7 weeks of maturation (Table 29). In batches 4 and 6, *L.monocytogenes* was not detected by direct plating but low numbers were detectable by enrichment broth culture at weeks 2 and 5 in batch 4 Camembert. In Camembert made from milk batch 6, the salt content was high (double the level of the previous cheeses, results not included) and this may have contributed to the lack of survival of *L.monocytogenes* in cheese made from milk thermised at 65.5°C (Table 30).

Camembert cheese made from milk thermised at 65.6°C had a lower level of surviving *L.monocytogenes* than cheese made from milk thermised at 62.8°C. However, both thermisation protocols resulted in cheeses that contained surviving *L.monocytogenes* which were capable of multiplication in Camembert cheese. It was found that LEB and LRB were generally equally capable of recovery of *L.monocytogenes* after thermisation treatments and from cheese but LRB tended to give higher recoverable counts. For further experiments, only LRB was used as an enrichment broth procedure.

7.3.3 DANISH BLUE CHEESE MADE FROM THERMISED MILK

Results for survival of *L.monocytogenes* in Danish blue cheese are shown in Tables 31-33. The counts of *L.monocytogenes* in milk batches 7-9 were between $1.75x10^2$-$5.4x10^3$ cfu/ml on inoculation and between $2.0x10^2$-$6.6x10^3$ cfu/ml after storage for 3 days at 4^oC. Danish blue cheese made from raw milk inoculated with *L.monocytogenes* contained high numbers of the organism detectable by direct plating for up to 7 weeks for batch 7 (Table 31) and 4 weeks for batch 8 and 9 cheeses (Tables 32 and 33). There was some initial multiplication during this period, after which numbers declined. *L.monocytogenes* was still recoverable after 7 or 9 weeks but not by 11 weeks. No *L.monocytogenes* was recovered from any of the cheeses made from milk thermised at 62.8 or 65.5^oC over the sampling time by enrichment procedures.

These results indicate that Danish blue cheese was the cheese type least capable of supporting survival and growth of *L.monocytogenes* amongst the 3 cheese types studied. This was probably because of the low pH (compared to Camembert and Canadian cheddar) and low water activity due to a combination of high salt and low moisture content (results not included).

All cheese composition for each batch are available from Dr Jean Banks, Hannah Research Institute, Ayr. This work was outwith the scope of this study.

7.3.4 EFFECT OF THERMISATION AND SUBSEQUENT PASTEURISATION OF *L.MONOCYTOGENES* IN MILK

Results of thermisation and subsequent pasteurisation of *L.monocytogenes* in milk are shown in Tables 34-39. The inoculum did not multipy after storage for 24 h, nor did it grow in the unthermised milk control after further storage for up to 48 h.

No *L.monocytogenes* was recovered from either batch of milk post-thermisation at 65.6°C for any time interval with an initial inoculum level of 10^3 cfu/ml of *L.monocytogenes* in milk (Tables 34-36. Consequently, there was no surviving *L.monocytogenes* to compromise HTST pasteurisation and no *L.monocytogenes* was recovered after 3 days' storage. In further experiments using a higher inoculum level of approximately 10^6 cfu/ml, thermisation temperature of 65.6°C (Tables 37-38) and 62.8°C (Table 39) were tested. Even at such a high inoculum level only a small number of organisms (recovered by enrichment culture) survived thermisation for 15 s on 2 occasions and once after 60 s (Tables 37-38). These small populations of survivors did not compromise HTST pasteurisation in any of the runs.

After thermisation at 62.8°C for 15 s *L.monocytogenes* could be detected by direct plating (Table 39) and was recovered after enrichment culture in milk thermised at 60 s and 120 s at 62.8°C. None of these populations grew over the 48 h storage period and no organisms survived HTST pasteurisation. There was no evidence that thermisation treatments induced heat shock proteins or allow large populations of *L.monocytogenes* to survive and multiply which could compromise HTST pasteurisation.

7.3.5 INDUCTION OF HEAT RESISTANCE IN *L.MONOCYTOGENES*

Exposure of *L.monocytogenes* cultures to heat treatments of 43-48°C for up to 30 minutes has been shown to induce increased resistance to further heating at higher temperatures such as 62.8°C when compared to control cultures (Knabel *et al.*, 1990). Milk inoculated with *L.monocytogenes* was pre-treated at 48°C for 15 or 30 minutes (positive control) or was untreated (negative control). These samples were then given a further treatment at 60°C and 65°C. The positive control organisms were found to be more resistant to heating at both 60°C and 65°C than the negative control organisms (Figures 9 and 10). This suggests that resistance factors had been induced in the positive control organisms and confirmed the findings of others (Fedio and Jackson, 1989 and Knabel *et al.*, 1990).

7.4 DISCUSSION

It was evident that *L.monocytogenes* in raw milk could survive and even multiply in long-hold Canadian cheddar cheese. High numbers of *L.monocytogenes* survived up to 9 months. Thermisation of milk at 62.8°C did not reduce numbers of *L.monocytogenes* substantially and could be recovered from this cheddar cheese up to the 9 months tested. Even after milk thermisation at 65.6°C, *L.monocytogenes* was recovered from cheddar cheese for up to 2 months. Thermisation, it was concluded, does not therefore ensure the absence of *L.monocytogenes* from this type of cheddar cheese if it is made with contaminated milk.

In studies with Camembert cheese, milk thermisation was even less

effective at removal of *L.monocytogenes* from this cheese than it was in cheddar cheese. Principally, this was because *L.monocytogenes* surviving thermisation could multiply to high levels in this soft cheese.

In Danish blue cheese made from raw milk containing *L.monocytogenes*, growth of the organism was not substantial over the maturation period and was absent by week 11. This was probably due to low water activity in this cheese. Danish blue cheese made from thermised milk did not allow survival of *L.monocytogenes* even when it was detected in milk post-thermisation. The surviving organisms were probably heat stressed and unable to recover in the adverse conditions of the cheese. Thermisation treatment may therefore be a sufficient pre-heat treatment for milk intended for this type of cheese manufacture.

Thermisation should obviously not be generally considered as an alternative to pasteurisation. *L.monocytogenes* was found to survive thermisation treatments of 62.8°C and 65.5°C for 16 s and be recoverable in subsequent cheese products. Thermisation does not however, compromise subsequent HTST pasteurisation by inducing tolerance to pasteurisation in *L.monocytogenes*.

FIGURE 8. Procedure for *L.monocytogenes* inoculated into raw milk, thermised and subsequently pasteurused

	treatment															
	10^3 cfu/ml (50 litres)								10^6 cfu/ml (50 litres)							
Procedure	Store at 4°C for 24 hours								Store at 4°C for 24 hours							
Thermise 65.6° or 62.8°C	15s		60s		120s		0s		15s		60s		120s		0s	
Store for 24 or 48 h at 4°C and pasteurise HTST 72°C/15s	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	(LITRES)								(LITRES)							
Store at 4°C up to 72 hours	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	(LITRES)								(LITRES)							

Table 25. *Listeria* recovered from batch 1 thermised milk and subsequent long hold Canadian-type cheddar

THERMISED MILK

Culture method	62.8°C	65.6°C
DP	0	0
LEB	+(2.03 x 10^3)	0
LRB	+(1.27 x 10^5)	0

CHEESE CURD

Sample time	Culture method	RAW	62.8°C	65.6°C
24 hour	DP	7.25 x 10^3	0	0
	LEB	+(1.53 x 10^6)	+(8.13 x 10^3)	0
	LRB	+(2.79 x 10^7)	+(5.84 x 10^6)	+(1.35 x10^6)

CHEESE

2 weeks	DP	5 x 10^4	0	0
	LEB	+(1.86 x 10^6)	+(6.7 x 10^4)	+(2.03 x10^2)
	LRB	+(2.64 x 10^6)	+(6.1 x 10^4)	+(2.5 x 10^2)
1 month	DP	9 x 10^4	0	0
	LEB	+(6.1 x 10^6)	+(4.88 x 10^3)	+(1.42 x10^4)
	LRB	+(2.85 x 10^7)	+(4.07 x 10^5)	+(1.79 x10^6)
2 month	DP	9 x 10^4	0	0
	LEB	+(6.1 x 10^5)	+(1.02 x 10^3)	0
	LRB	+(1.02 x 10^6)	+(6.1 x 10^4)	+(8.1 x 10^3)
3 month	DP	3.80 x 10^4	0	0
	LEB	+(8.74 x 10^5)	+(8.13 x 10^2)	0
	LRB	+(2.44 x 10^6)	+(2.03 x 10^3)	0
4 month	DP	1 x 10^3	0	0
	LEB	+(6.71 x 10^5)	0	0
	LRB	+(2.24 x 10^5)	0	0
5 month	DP/LEB/LRB	ND	ND	ND

DP = direct plating on Oxford agar; LEB = *Listeria* enrichment broth; LRB = *Listeria* resuscitation broth;

\+ = *L. monocytogenes* recovered; figures in parenthesis are the actual count of *L.monocytogenes* recovered after enrichment or resuscitation broth culture.

0 = *L. monocytogenes* was not detected at the lowest limit possible which for direct plating was 1.0 cfu/ml and for enrichment culture meant absent in 25 g of sample.

ND = not determined.

Table 25 continued. ***Listeria*** **recovered from batch 1 thermised milk and subsequent long hold Canadian-type cheddar**

		CHEESE		
6 month	DP	1.4×10^4	0	0
	LEB	$+(6.10 \times 10^3)$	0	0
	LRB	$+(4.07 \times 10^6)$	0	0
7 month	DP	5.2×10^3	0	0
	LEB/LRB	+	0	0
8 month	DP	2.4×10^3	0	0
	LEB	$+(2.03 \times 10^3)$	0	0
	LRB	$+(1.63 \times 10^5)$	0	0
9 month	DP	2.0×10^3	0	0
	LEB	$+(3.05 \times 10^3)$	0	0
	LRB	$+(5.43 \times 10^5)$	0	0

DP = direct plating on Oxford agar; LEB = *Listeria* enrichment broth; LRB = *Listeria* resuscitation broth;
+ = *L. monocytogenes* recovered; figures in parenthesis are the actual count of *L.monocytogenes* recovered after enrichment or resuscitation broth culture.
0 = *L. monocytogenes* was not detected at the lowest limit possible which for direct plating was 1.0 cfu/ml and for enrichment culture meant absent in 25 g of sample.
ND = not determined.

Table 26. *Listeria* recovered from batch 2 thermised milk and subsequent long hold Canadian Cheddar

THERMISED MILK

Culture method	62.8°C	65.6°C
DP	1×10^3	0
LEB	$+(4.26 \times 10^9)$	$+(8.13 \times 10^2)$
LRB	$+(5 \times 10^9)$	$+(1.02 \times 10^2)$

CHEESE CURD

Sample time	Culture method	RAW	62.8°C	65.6°C
24 hour	DP	3.2×10^5	3.2×10^5	10
	LEB	$+(1.02 \times 10^7)$	$+(1.02 \times 10^6)$	$+(9.09 \times 10^6)$
	LRB	$+(1.02 \times 10^7)$	$+(1.72 \times 10^6)$	$+(1.12 \times 10^6$

CHEESE

2 weeks	DP	2×10^5	9×10^4	60
	LEB	$+(3.9 \times 10^6)$	$+(2.9 \times 10^6)$	$+(3.25 \times 10^6)$
	LRB	$+(1.5 \times 10^7)$	$+(1.2 \times 10^7)$	$+(2.3 \times 10^5)$
1 month	DP	3×10^5	3.7×10^4	0
	LEB	$+(1.2 \times 10^7)$	$+(6.7 \times 10^6)$	$+(6.5 \times 10^7)$
	LRB	$+(1.5 \times 10^8)$	$+(3.25 \times 10^6)$	$+(8.1 \times 10^5)$
2 month	DP	2×10^5	5×10^3	0
	LEB/LRB	ND	ND	ND
3 month	DP	2.1×10^5	1.5×10^4	0
	LEB	$+(1.63 \times 10^6)$	+	+
	LRB	$+(4.47 \times 10^7)$	$+(8.13 \times 10^6)$	$+(1.22 \times 10^6)$
4 month	DP	1.4×10^4	3.00×10^2	0
	LEB	$+(4.09 \times 10^5)$	$+(3.86 \times 10^3)$	$+(6.1 \times 10^4)$
	LRB	$+(4.31 \times 10^7)$	$+(2.03 \times 10^6)$	$+(1.81 \times 10^7)$
5 month	DP/LEB/LRB	ND	ND	ND
6 month	DP	1.08×10^5	7×10^2	0
	LEB	$+(2.67 \times 10^4)$	0	0
	LRB	$+(5.47 \times 10^7)$	$+(3.86 \times 10^4)$	$+(1.17 \times 10^5)$
7 month	DP	5.5×10^4	90	0
	LEB	$+(1.02 \times 10^5)$	0	0
	LRB	$+(1.31 \times 10^7)$	$+(1.3 \times 10^5)$	$+(3.28 \times 10^4)$
8 month	DP	4.0×10^4	60	0
	LEB	$+(3.06 \times 10^5)$	0	0
	LRB	$+(2.24 \times 10^6)$	$+(3.52 \times 10^5)$	0
9 month	DP	2.30×10^4	0	0
	LEB	$+(7.72 \times 10^5)$	0	0
	LRB	$+(6.65 \times 10^6)$	$+(1.02 \times 10^4)$	0

Legends as in Table 25.

Table 27. *Listeria* recovered from batch 3 thermised milk and subsequent long hold Canadian Cheddar

THERMISED MILK

Culture method	62.8°C	65.6°C
DP	5.53×10^2	0
LEB	$+(3.52 \times 10^9)$	$+(2.24 \times 10^3)$
LRB	$+(1.2 \times 10^{10})$	$+(2.96 \times 10^9)$

CHEESE CURD

Sample time	Culture method	RAW	62.8°C	65.6°C
24 hour	DP	1.5×10^5	1.8×10^3	0
	LEB	$+(2.03 \times 10^6)$	$+(4.38 \times 10^5)$	$+(8.13 \times 10^3)$
	LRB	$+(8.13 \times 10^6)$	$+(1.83 \times 10^7)$	$+(1.2 \times 10^5)$

CHEESE

2 week	DP	5.3×10^4	1.8×10^3	0
	LEB	$+(2.4 \times 10^7)$	$+(2.8 \times 10^7)$	0
	LRB	$+(7.7 \times 10^6)$	$+(2.8 \times 10^7)$	0
1 Month	DP	9×10^4	2.3×10^3	0
	LEB	$+(9.6 \times 10^6)$	$+(2.2 \times 10^5)$	0
	LRB	$+(2 \times 10^8)$	$+(6.5 \times 10^6)$	0
2 month	DP	5.2×10^4	1.4×10^3	0
	LEB	$+(3.46 \times 10^6)$	$+(9.35 \times 10^6)$	$+(2.03 \times 10^3)$
	LRB	$+(1.18 \times 10^8)$	$+(1.06 \times 10^7)$	$+(2.03 \times 10^3)$
3 month	DP	9.4×10^3	3.0×10^2	0
	LEB	$+(1.46 \times 10^7)$	$+(8.13 \times 10^5)$	0
	LRB	$+(1.86 \times 10^7)$	$+(2.93 \times 10^6)$	0
4 month	DP/LEB/LRB	ND	ND	ND
5 month	DP	6.3×10^4	9.1×10^2	0
	LEB	$+(2.03 \times 10^5)$	0	0
	LRB	$+(2.65 \times 10^7)$	$+(6.57 \times 10^5)$	0
6 month	DP	2.9×10^4	4.6×10^2	0
	LEB	+	+	0
	LRB	+	+	0
7 month	DP	3.3×10^4	1.20×10^2	0
	LEB	$+(1.63 \times 10^5)$	0	0
	LRB	$+(3.06 \times 10^7)$	$+(2.03 \times 10^5)$	0
8 month	DP	2.4×10^4	50	0
	LEB	$+(1.63 \times 10^5)$	0	0
	LRB	ND	ND	ND

Legends as in Table 25.

Table 28. *Listeria* recovered from batch 7 thermised milk and subsequent Camembert cheese

THERMISED MILK

Culture method	62.8°C	65.6°C
DP	0	0
LEB	0	0
LRB	+(1.22×10^4)	+(2.85×10^4)

CHEESE CURD

Sample time	Culture method	RAW	62.8°C	65.6°C
24 hour	DP	2.4×10^4	0	0
	LEB	+(7.11×10^4)	0	0
	LRB	+(1.83×10^4)	+(1.02×10^3)	0

CHEESE

1 week	DP	5.6×10^4	30	0
	LEB	+(2.7×10^6)	+(6.1×10^2)	0
	LRB	+(1.42×10^6)	+(2.03×10^3)	0
2 week	DP	7.6×10^4	60	0
	LEB	+(1.48×10^5)	+(4.67×10^3)	+(8.13×10^2)
	LRB	+(1.22×10^4)	+(3.66×10^4)	+(4.07×10^3)
5 week	DP	9.8×10^5)	1.2×10^2)	0
	LEB	+(1.05×10^7)	+(4.07×10^2)	0
	LRB	+(1.44×10^5)	+(6.09×10^2)	+(2.24×10^3)
7 week	DP	7.8×10^5	6.4×10^3	0
	LEB	+	+	0
	LRB	+(1.41×10^6)	+(2.48×10^6)	0

Legends as in Table 25.

Table 29. ***Listeria*** **recovered from batch 8 thermised milk and subsequent Camembert cheese**

THERMISED MILK

Culture method	62.8°C	65.6°C
DP	27	0
LEB	+(8.54×10^5)	+(4.67×10^5)
LRB	+($>1.85 \times 10^8$)	+(2.11×10^6)

CHEESE CURD

Sample time	Culture method	RAW	62.8°C	65.6°C
24 hour	DP	8.6×10^4	60	0
	LEB	+(1.63×10^4)	+(2.03×10^3)	0
	LRB	+(2.03×10^2)	+(6.10×10^4)	0

CHEESE

1 week	DP	3.6×10^5	60	0
	LEB	+(5.69×10^5)	0	0
	LRB	+(3.05×10^3)	+(1.04×10^5)	0
2 week	DP	8×10^4	60	40
	LEB	+(5.89×10^5)	+(1.04×10^5)	+(7.32×10^3)
	LRB	ND	ND	ND
5 week	DP	1.18×10^5	+	+
	LEB	+(7.95×10^5)	+(7.52×10^5)	+(2.23×10^4)
	LRB	+(2.4×10^6)	+(7.11×10^4)	+(1.02×10^3)
7 week	DP	1.90×10^6	9.4×10^3	1×10^2
	LEB	ND	ND	ND
	LRB	ND	ND	ND

Legends as in Table 25.

Table 30. *Listeria* **recovered from batch 9 thermised milk and subsequent Camembert cheese**

THERMISED MILK

Culture method	62.8°C	65.6°C
DP	0	0
LEB	ND	ND
LRB	ND	ND

CHEESE CURD

Sample time	Culture method	RAW	62.8°C	65.6°C
24 hour	DP	1.8×10^4	0	0
	LEB	$+(6.5 \times 10^6)$	0	0
	LRB	$+(3.75 \times 10^7)$	0	0

CHEESE

2 week	DP	1.06×10^5	0	0
	LEB	$+(7.72 \times 10^4)$	0	0
	LRB	$+(3.05 \times 10^4)$	0	0
5 week	DP	1.1×10^6	6.1×10^3	0
	LEB	ND	ND	ND
	LRB	$+(1.09 \times 10^7)$	$+(7.45 \times 10^3)$	0
7 week	DP	2.55×10^6	4×10^3	0
	LEB/LRB	ND	ND	ND

Legends as in Table 25.

Table 31. ***Listeria*** **recovered from batch 10 thermised milk and subsequent Danish Blue cheese**

THERMISED MILK

Culture method	62.8°C	65.6°C
DP	0	0
LEB	0	0
LRB	0	0

CHEESE CURD

Sample time	Culture method	RAW	62.8°C	65.6°C
24 hour	DP	1×10^2	0	0
	LRB	$+(1 \times 10^4)$	0	0

CHEESE

1 week	DP	5×10^2	0	0
	LRB	$+(4.07 \times 10^4)$	0	0
2 week	DP	8×10^2	0	0
	LRB	$+(4.07 \times 10^3)$	0	0
4 week	DP	1.02×10^3	0	0
	LRB	$+(8.03 \times 10^4)$	0	0
7 week	DP	8×10^2	0	0
	LRB	+	0	0
9 week	DP	0	0	0
	LRB	0	0	0

Legends as in Table 25.

Table 32. *Listeria* recovered from batch 11 thermised milk and subsequent Danish Blue cheese

THERMISED MILK

Culture method	62.8°C	65.6°C
DP	28	0
LEB	0	0
LRB	+(2.03×10^4)	0

CHEESE CURD

Sample time	Culture method	RAW	62.8°C	65.6°C
24 hour	DP	4×10^4	0	0
	LRB	+(4×10^5)	0	0

CHEESE

1 week	DP	1×10^3	0	0
	LRB	+(3.5×10^3)	0	0
2 week	DP	+	0	0
	LRB	+(2.03×10^2)	0	0
4 week	DP	6.10×10^2	0	0
	LRB	+(4.07×10^4)	0	0
7 week	DP	ND	ND	ND
	LRB	ND	ND	ND
9 week	DP	0	0	0
	LRB	+(3.66×10^5)	0	0

Legends as in Table 25.

Table 33. *Listeria* recovered from batch 12 thermised milk and subsequent Danish Blue cheese

THERMISED MILK

Culture method	62.8°C	65.6°C
DP	1	0
LEB	0	0
LRB	+(1.28×10^9)	0

CHEESE CURD

Sample time	Culture method	RAW	62.8°C	65.6°C
24 hour	DP	0	0	0
	LRB	ND	ND	ND

CHEESE

1 week	DP	1×10^3	0	0
	LRB	+(2.03×10^3)	0	0
2 week	DP	2×10^2	0	0
	LRB	ND	0	0
4 week	DP	60	0	0
	LRB	+(2.03×10^2)	0	0
7 week	DP	0	0	0
	LRB	+(8.94×10^3)	0	0
9 week	DP	ND	ND	ND
	LRB	ND	ND	ND
11 week	DP	0	0	0
	LRB	0	0	0

Legends as in Table 25.

Table 34. Recovery of *Listeria monocytogenes* after various thermisation, storage and pasteurisation treatments in milk batch A

	Pre-thermisation	Post-thermisation									
		After 24 hours' storage					After 48 hours' storage				
Thermisation (65.6°C) time (seconds)	Counts (cfu/ml) after 24 hours 4°C storage	Pre-HTST	Post-HTST (hours)				Pre-HTST	Post-HTST (hours)			
			0	24	48	72		0	24	48	72
0	2.7×10^3	2.4×10^3	0	0	0	0	2.2×10^3	0	0	0	0
15	2.7×10^3	0	0	0	0	0	0	0	0	0	0
60	2.7×10^3	0	0	0	0	0	0	0	0	0	0
120	2.7×10^3	0	0	0	0	0	0	0	0	0	0

Legends as in Table 25.

Table 35. Recovery of *Listeria monocytogenes* after various thermisation, storage and pasteurisation treatments in milk batch B

Thermisation (65.6°C) time (seconds)	Pre-thermisation	Post-thermisation									
	Counts (cfu/ml) after 24 hours 4°C storage	After 24 hours' storage					After 48 hours' storage				
		Pre-HTST	Post-HTST (hours)				Pre-HTST	Post-HTST (hours)			
			0	24	48	72		0	24	48	72
0	3.0×10^3	7.0×10^3	0	0	0	0	4.3×10^3	0	0	0	0
15	3.0×10^3	0	0	0	0	0	0	0	0	0	0
60	3.0×10^3	0	0	0	0	0	0	0	0	0	0
120	3.0×10^3	0	0	0	0	0	0	0	0	0	0

Legends as in Table 25.

Table 36. Recovery of *Listeria monocytogenes* after various thermisation, storage and pasteurisation treatments in milk batch C

	Pre-thermisation	Post-thermisation									
		After 24 hours' storage					After 48 hours' storage				
Thermisation (65.6°C) time (seconds)	Counts (cfu/ml) after 24 hours 4°C storage	Pre-HTST	Post-HTST (hours)				Pre-HTST	Post-HTST (hours)			
			0	24	48	72		0	24	48	72
0	2.77×10^4	5.74×10^4	0	0	0	0	6.57×10^4	0	0	0	0
15	2.77×10^4	0	0	0	0	0	0	0	0	0	0
60	2.77×10^4	0	0	0	0	0	0	0	0	0	0
120	2.77×10^4	0	0	0	0	0	0	0	0	0	0

Legends as in Table 25.

Table 37. Recovery of *Listeria monocytogenes* after various thermisation, storage and pasteurisation treatments in milk batch D

	Pre-thermisation	Post-thermisation									
		After 24 hours' storage					After 48 hours' storage				
Thermisation (65.6°C) time (seconds)	Counts (cfu/ml) after 24 hours 4°C storage	Pre-HTST	Post-HTST (hours)				Pre-HTST	Post-HTST (hours)			
			0	24	48	72		0	24	48	72
0	1.17×10^6	6.67×10^5	0	0	0	0	5.69×10^5	0	0	0	0
15	1.17×10^6	+	0	0	0	0	0	0	0	0	0
60	1.17×10^6	0	0	0	0	0	0	0	0	0	0
120	1.17×10^6	0	0	0	0	0	0	0	0	0	0

Legends as in Table 25.

Table 38. Recovery of *Listeria monocytogenes* after various thermisation, storage and pasteurisation treatments in milk batch E

	Pre-thermisation	Post-thermisation									
		After 24 hours' storage					After 48 hours' storage				
Thermisation (65.6°C) time (seconds)	Counts (cfu/ml) after 24 hours 4°C storage	Pre-HTST	Post-HTST (hours)				Pre-HTST	Post-HTST (hours)			
			0	24	48	72		0	24	48	72
0	7.95×10^5	1.06×10^6	0	0	0	0	1.82×10^5	0	0	0	0
15	7.95×10^5	+	0	0	0	0	+	0	0	0	0
60	7.95×10^5	+	0	0	0	0	0	0	0	0	0
120	7.95×10^5	0	0	0	0	0	0	0	0	0	0

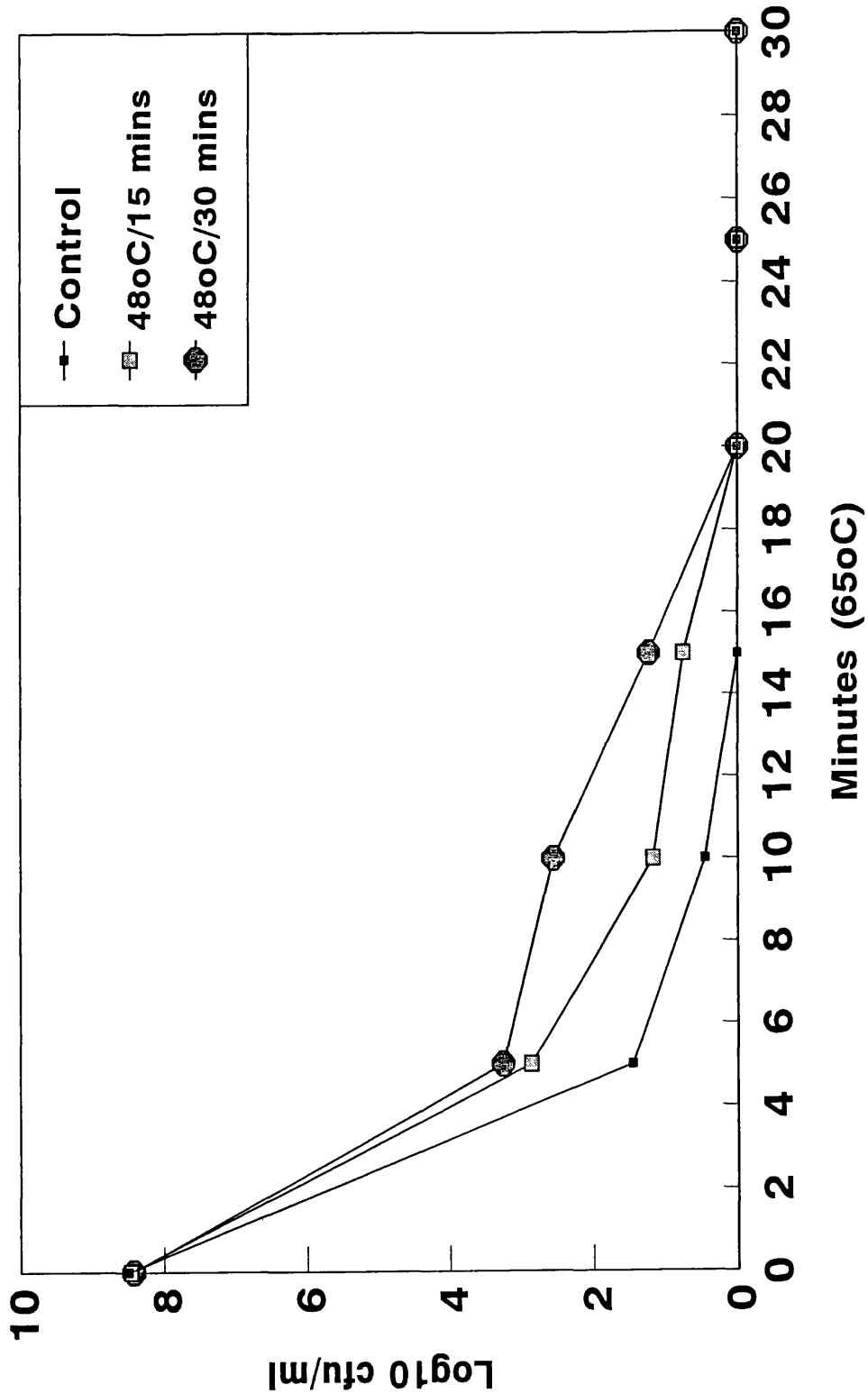
Legends as in Table 25.

Table 39. Recovery of *Listeria monocytogenes* after various thermisation, storage and pasteurisation treatments in milk batch F

	Pre-thermisation	Post-thermisation									
		After 24 hours' storage					After 48 hours' storage				
Thermisation (62.8°C) time (seconds)	Counts (cfu/ml) after 24 hours 4°C storage	Pre-HTST	Post-HTST (hours)				Pre-HTST	Post-HTST (hours)			
			0	24	48	72		0	24	48	72
0	8.1×10^5	1.59×10^6	0	0	0	0	2.12×10^6	0	0	0	0
15	8.1×10^5	2.06×10^4	0	0	0	0	2×10^3	0	0	0	0
60	8.1×10^5	+	0	0	0	0	+	0	0	0	0
120	8.1×10^5	+	0	0	0	0	+	0	0	0	0

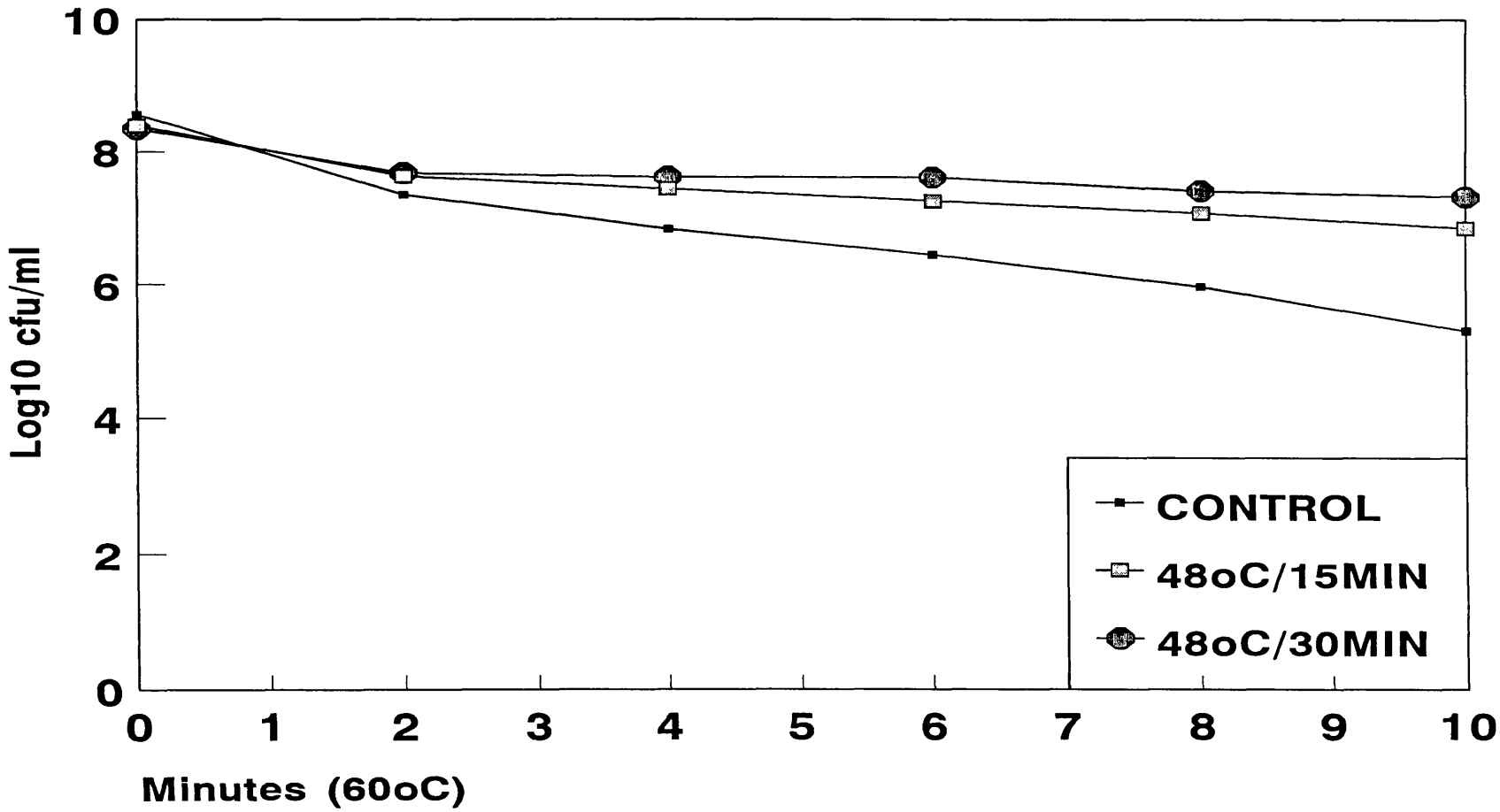
Legends as in Table 25.

FIGURE 9 INDUCTION OF HEAT RESISTANCE IN LISTERIA MONOCYTOGENES(SCOTT A) AT 65oC*



* Mean of 3 trials

FIGURE 10 INDUCTION OF HEAT RESISTANCE IN LISTERIA MONOCYTOGENES(SCOTT A)*



* MEAN OF 3 TRIALS

CHAPTER 8

THE GENERATION TIMES OF *LISTERIA MONOCYTOGENES* AND *LISTERIA INNOCUA* IN TWO *LISTERIA* ENRICHMENT BROTHS

8.1 INTRODUCTION

Most species of the genus *Listeria* have been isolated from raw cows' milk (Lovett *et al.*,1987; Rea *et al.*, 1992; Harvey and Gilmour, 1992), the most frequently isolated being *L.innocua* and *L.monocytogenes.* Since *Listeria* spp. are generally present in low numbers, isolation of *Listeria* spp. from milk is usually only successful after enrichment procedures. Harvey and Gilmour (1992), for instance, never isolated *L.monocytogenes* by direct plating of 176 raw milk samples but isolated *L.monocytogenes* and *L.innocua* by enrichment procedures from 15.3 and 10.2% of samples respectively.

From the results in Chapter 3, it was found that the incidence of *L.monocytogenes* in raw sheep, cow and goat milk was considerably lower than the incidence of *L.innocua.* On no occasion were both species isolated from the same sample after enrichment broth culture. The presence of *Listeria* spp. in samples after incubation in enrichment broth is usually shown by streaking a loopful of the broth onto a selective agar and selecting 5 presumptive *Listeria* colonies for further identification (Anon, 1990). If there were any differences in growth rate of the two species in enrichment broth then it would be likely that only the faster growing species would be recovered after enrichment broth culture. This might be at least one reason why *L.innocua* is more frequently isolated from milk samples than *L.monocytogenes.*

Experiments were designed therefore to determine whether *L.innocua* could outgrow *L.monocytogenes* when cultured either separately or in mixed culture in two selective *Listeria* enrichment broths. Generation times for six strains each of these *Listeria* spp. were calculated by impedance techniques and confirmed for one strain of each species by conventional plating methods.

8.2 EXPERIMENTAL PROCEDURES

8.2.1 CULTURES

Listeria monocytogenes strains used were Scott A, L519, L527, L533, L560, and L580 (see section 2.1.1). *L.innocua* strains used were NCTC 11288, L510, L523, L534, L553 and L585 (see section 2.1.1).

8.2.2 *LISTERIA* ENRICHMENT BROTHS

The *Listeria* enrichment broths used in this study were LEB and LRB (see section 2.1.4).

8.2.3 IMPEDANCE STUDY

A Bactometer Model M64 (BioMérieux UK Ltd) was used with disposable module cassettes (see section 2.2.2). Duplicate wells were filled with 1 ml of either TSB, LEB or LRB. These wells were inoculated in duplicate with 10 µl volumes of either a 10^{-6} or 10^{-4} dilution of a single strain of *L.monocytogenes* or *L.innocua* strain. The cassettes were incubated at 30°C for 50 h and impedance measured as capacitance was monitored throughout.

By recording the differences in detection time (DT) between the 10^{-6}

and 10^{-4} dilutions of the initial inoculum, the generation time (tg) for each *Listeria* spp. was calculated using the following equation (Eden and Eden, 1984):

$$tg = \Delta IDT \times \log 2 / \log_{10}(N1) - \log_{10}(N2)$$

$$\text{Since } \log_{10}(N1) - \log_{10}(N2) = 2 \text{ and } \log 2 = 0.301$$

$$\text{then } tg = 0.15 \times \Delta IDT$$

where ΔIDT = the difference in initial detection time between the highest and lowest dilutions of inoculum

$\log_{10}(N1)$ = log count of the lowest dilution of inoculum

$\log_{10}(N2)$ = log count of the highest dilution of inoculum

8.2.4 PLATE COUNT STUDY

30 ml of TSB, LEB and LRB were inoculated with 30 µl of a 10^{-5} dilution of a 24 h TSB culture of *L.monocytogenes* (Scott A) or *L.innocua* (NCTC 11288) and incubated at 30°C for 24 h. Every two hours 2x500 µl samples (with appropriate dilutions in MRD) (see section 2.1.4) were spread plated (Stainer *et al.*, 1987) or, for later samples, spiral plated using a Spiral Plater Model D (Spiral Systems Inc) (see section 2.2.1) onto duplicate Oxford agar plates (see section 2.1.4). All plates were incubated at 37°C for 48 h. All plates were counted using a Plate Counter (Spiral Systems Inc) (see section 2.2.1) to calculate cfu/ml.

The tg of microbial population was determined using conventional plate count and calculating the growth rate constant (k) from the exponential growth curve (Stainer *et al.*, 1987) determined for *L.monocytogenes* and *L.innocua* where:

$$k = (\log_{10}Z - \log_{10}Z_o)\ 2.303 / t - t_o$$

From this, tg was calculated by

$$k = \log_e 2 / tg \text{ and so } tg = 0.693 / k$$

where Z = colony forming units/ml at t (time)

Z_o = colony forming units/ml at t_o (time o)

8.2.5 FATE OF *LISTERIA MONOCYTOGENES* AND *LISTERIA INNOCUA* IN MIXED CULTURE

L.monocytogenes (Scott A) and *L.innocua* (NCTC 11288) were simultaneously inoculated into 225 ml LEB to yield approximately 10 cfu/ml of each species. The enrichment broth was incubated at 30°C for 48 h and then a loopful streaked onto duplicate Oxford agar plates (Anon, 1990). After incubation at 37°C for 24 h, eight presumptive *Listeria* colonies were selected from Oxford agar for identification (see section 2.2.3). Differentiation between the species was achieved using the API *Listeria* test strip (BioMérieux UK Ltd). This test strip differentiates *L.monocytogenes* and *L.innocua* on the basis of the absence of arylamidase from the former (Bille *et al*, 1992), *ie.* helps differentiate between weakly haemolytic *L.monocytogenes* and non-haemolytic *L.innocua*.

8.3 RESULTS

The generation times between *L.monocytogenes* and *L.innocua* in TSB (non-specific broth) and LRB were not significantly different (Table 40). The generation times for *L.monocytogenes* and *L.innocua* strains in LEB were significantly different ($p<0.001$) with *L.innocua* having the faster generation time (Table 40).

These results prompted the next experiment wherein *L.monocytogenes*

(Scott A) and *L.innocua* (NCTC 11288) were simultaneously inoculated into 225 ml LEB to yield approximately 10 cfu/ml of each species. It was found that all of the eight randomly selected colonies isolated from streak plates of incubated LEB were identified as *L.innocua* (API *Listeria* code 7510). *L.monocytogenes* was not detected.

Similar growth patterns were obtained for *L.monocytogenes* (Scott A) and *L.innocua* (NCTC 11288) using conventional plating compared to impedance methods but in all cases calculated generation times were faster using impedance (Table 41). The difference in generation times between the two methods may be due to impedance relying on the measurement of metabolic changes, whereas conventional plating depends on the production of a visible biomass.

8.4 DISCUSSION

Very recently Petran and Swanson (1993) have also reported a difference in the growth rate of *L.monocytogenes* and *L.innocua* but these authors used Fraser Broth (FB) and University of Vermont (UVM) *Listeria* enrichment broth. Previously, Duh and Schaffer (1993) have shown *L.innocua* grows faster than *L.monocytogenes* at temperatures below 42°C, but in the studies reported here no significant differences in the growth rate of these species was shown in TSB at 30°C using impedance measured as capacitance (Table 40).

It was found that when both *L.monocytogenes* and *L.innocua* were grown together in LEB, *L.innocua* outgrew *L.monocytogenes.* The findings shown here indicate that if a milk or food sample containing *L.innocua* and *L.monocytogenes*

was examined using LEB as the selective enrichment broth it could result in *L.monocytogenes* being undetected.

This possibility was not tested using a milk sample artificially contaminated with equal numbers of *L.monocytogenes* and *L.innocua* but Petran and Swanson (1993) have since shown that only *L.innocua* was isolated from a cheese sauce artificially contaminated with equal numbers of *L.monocytogenes* and *L.innocua* after enrichment procedures using UVM and FB. These results are in agreement with the results of this chapter using LEB.

At present, the International Dairy Federation (Anon, 1990) recommends the use of LEB for detection of *L.monocytogenes* in milk and milk products by enrichment procedures. These experiments show that using LEB as a selective enrichment broth for the isolation of *L.monocytogenes* from a food sample may result in this organism not being recovered if significant levels of *L.innocua* are also present.

TABLE 40. Mean generation times of *Listeria monocytogenes* and *Listeria innocua* in TSB, LEB and LRB using impedance technology

Bacterial Strain	Generation time (minutes) TSB	LEB	LRB
L.monocytogenes			
Scott A	44.40	78.60	61.50
L519	40.95	68.40	55.35
L527	33.75	62.55	51.75
L533	49.95	72.90	50.40
L560	40.50	67.05	53.10
L580	38.25	67.95	55.80
Grand mean	42.92	71.93	50.23
(±S.E.M)	1.12	3.62	2.54
L.innocua			
NCTC 11288	43.65	52.50	53.10
L510	41.40	53.10	60.75
L523	53.55	56.25	49.05
L534	41.85	59.85	43.65
L553	38.25	56.70	46.80
L585	39.15	55.35	50.40
Grand mean	43.03	54.84	51.24
(±S.E.M)	1.78	1.46	2.11

TABLE 41. Generation times of *Listeria monocytogenes* (Scott A) and *Listeria innocua* (NCTC 11288) in broths using impedance technology and plate count methods

	Impedance method		Plate count method	
Broth	*L.monocytogenes*	*L.innocua*	*L.monocytogenes*	*L.innocua*
TSB	44.40	43.65	54.50	50.10
LEB	78.60	52.50	94.56	77.85
LRB	61.50	53.10	79.34	75.85

CHAPTER 9

GENERAL DISCUSSION

The results of the survey of the incidence of *L.monocytogenes* in milk and cheese showed that raw sheep, goat and cow milk all frequently contain *L.monocytogenes* whereas the organism was only ever isolated from a single cheese type produced using raw sheep milk. The overall incidence of *Listeria* spp. in raw cow milk from dairy silos was 32.3%, with *L.monocytogenes*, *L.innocua* and *L.seeligeri* at 8.9%, 21.1% and 2.2% respectively. Other studies have reported a much higher incidence of *Listeria* spp. and for *L.monocytogenes* (Harvey and Gilmour, 1992; Dominguez-Rodriguez *et al.*, 1985; Fernandez-Garayzabal *et al.*.,1987). This could reflect the high quality achieved by Scottish farms and dairies compared with other countries.

The source of contamination with *L.monocytogenes* could be from the soil (Welshimer and Donket-Voet, 1971) with the organism getting onto the udders during milking and being tranferred into the milk. Silage has also been shown to be a reservoir for *L.monocytogenes*, particularly poor quality mouldy silage (Fenlon, 1985). Silage was implicated in a suspected outbreak of listeriosis in calves (Fenlon, 1986). Ingestion of contaminated silage may result in excretion of *L.monocytogenes* in the faeces which consequently may infect pasture in a cycle of infection. *L.monocytogenes* can be shed in both the milk and faeces of cows (Fedio and Jackson, 1992). These reservoirs of *L.monocytogenes* in the environment and in the animal population allows easy access for this organism into food products.

In this survey, *L.monocytogenes* and *L.innocua* were isolated in raw sheep milk at 4.8% and 19% and in goat milk at 1.9% and 3.7%, respectively. These results suggest that using these milks without prior heat treatment for a manufactured dairy product could result in the finished product containing *L.monocytogenes* since the growth kinetics of *L.monocytogenes* in many dairy products (Chapter 5; Ryser and Marth, 1987a; Ryser and Marth, 1987b; Papageorgiou and Marth, 1989; Dominguez *et al.*, 1987; Ryser *et al.*, 1985; Choi *et al.*, 1988) have shown that the organism can readily grow and survive in these products.

The overall incidence of *Listeria* spp. isolated from cheese samples over 1 year (Chapter 3) was 4.9%, with *L.monocytogenes* and *L.innocua* isolated at 1% and 3.9% respectively. Correlation between total bacterial count and isolation of *Listeria* spp. was studied but no information was concluded as the corrolation should have been with total *Enterobacteriaceae* since the cheeses used were fermented so would contain lactic acid bacteria from the starter culture.

Hazard analysis critical control points (HACCP) in cheese production facilities should include the cheese ripening rooms since dirty storage shelves, floor, ceiling etc. could be contaminated with *L.monocytogenes* and result in a final product being contaminated. All these areas should be manufactured from non-porous material such as stainless steel to allow for adequate cleaning. In the listeriosis outbreak involving Vacherin Mont d'Or soft cheece, the epidemic strain of *L.monocytogenes* was isolated from the shelves in the cheese ripening rooms (Bille, 1990). It is therefore essential that mould-ripened cheese be tested for the presence of *L.monocytogenes* at the end of the ripening period just

before leaving the production facility as post-manufacture contamination with *L.monocytogenes* can occur. This was shown in this study with Camembert cheese (Chapter 5), even at low levels of *L.monocytogenes* being contaminated onto the cheese at 2 weeks resulted in a cheese with a high final count of *L.monocytogenes* at levels of 10^3- 10^5cfu/g. Recently, *L.monocytogenes* was found in a Blue-veined cheese manufactured from raw sheep milk (Anon, 1995). This cheese was shown in this study to be unable to support the growth of *L.monocytogenes* when inoculated into the raw milk at a level of $3x10^4$ cfu/ml and was not found to survive in the finished product. It would seem likely that the source of contamination found in the cheese would be post-manufacture contamination. It was shown in this study (Chapter 5) that *P.camamberti* strain Niege previously shown to be inhibitory *in vitro* to *Listeria* spp. was also shown to be inhibitory to *L.monocytogenes* on the surface of the cheese. This suggests that selection of the ripening moulds for this characteristic could potentially reduce growth of *L.monocytogenes* in a post-manufacture contamination situation.

It was shown that the isolation of *L.innocua* in the milk and cheese samples tested in the survey occurred outwith the isolation of *L.monocytogenes.* Both *L.innocua* and *L.monocytogenes* were never isolated from the same sample simultaneously. In this survey the *Listeria* enrichment broth used was LEB followed by plating onto Oxford agar. Since the work in this thesis, it has been shown that *L.innocua* has a higher growth rate compared with *L.monocytogenes* when grown in Fraser Broth and University of Vermont (UVM) *Listeria* enrichment broths (Petran and Swanson, 1993). This was also shown

in this study using LEB (see Chapter 8) with *L.innocua* strains having a faster generation time than *L.monocytogenes* strains tested. A *Listeria* Repair Broth (LRB) was developed by Busch and Donnelly (1992) for resuscitation of heat-injured *L.monocytogenes* and *L.innocua*. It was shown in this study that no significant differences in the growth rates of *L.monocytogenes* and *L.innocua* were shown when grown in LRB. This would suggest that if equal numbers of *L.monocytogenes* and *L.innocua* were incubated in LRB, the isolates recovered after streak plating onto a suitable agar would be roughly equal numbers of *L.monocytogenes* and *L.innocua*. These results would seem to suggest that recovery of *L.monocytogenes* from food samples would be more likely using LRB compared with other commercially available *Listeria* enrichment broths.

Further work would be necessary to show that LRB was the better *Listeria* enrichment broth for the recovery of *L.monocytogenes* from food. This could be done by sampling a number of food products and comparing the recovery of *Listeria* spp. using LRB and other commercially available *Listeria* enrichment broths such as LEB, UVM broth and Fraser broth. This could also be done by artificially contaminating a range of food samples with varying levels of *L.monocytogenes* and *L.innocua* and comparing recovery in a range of *Listeria* enrichment broths.

From the results in Chapter 7, it was shown that LRB could recover *L.monocytogenes* equally well and in some cases better than LEB from cheese samples. In some instances, LRB recovered *L.monocytogenes* from the cheese samples while LEB gave a negative result after incubation. Since milk was

thermised and cheese subsequently manufactured from it, it would seem that LRB did repair and resuscitate heat-injured *L.monocytogenes* in these samples as suggested by Busch and Donnelly (1992). Higher counts of *L.monocytogenes* were recovered after enrichment culture using LRB than with LEB as total cfu/g were calculated in each sample after incubation. The reason for this difference in the recovery between LRB and LEB maybe due to the 5 hour incubation of LRB before the addition of the supplements whereas the supplements for LEB are added at the start of the incubation period. The base media are also different for these two *Listeria* enrichment broths (see section 2.1.4). LEB contains TSYEB whereas LRB contains TSYEB plus Glucose, MOPS (free acid), MOPS (sodium salt), Ferrous sulphate, Magnesium sulphate and pyruvate. By supplementing TSB with these compounds, Busch and Donnelly (1992) showed that the heat-injured *L.monocytogenes* and *L.innocua* strains used were completely repaired within 5 hours. They have also shown that higher levels of *L.monocytogenes* and *L.innocua* were shown after incubation compared with LEB, FDA broth and UVM broth using pure cultures. This has now also been shown in this study using cheese samples artificially contaminated with *L.monocytogenes* during manufacture.

At present, the International Dairy Federation (IDF) (Anon, 1990) recommends the use of LEB for detection of *L.monocytogenes* in milk and milk products by enrichment procedures. The results in this study suggest that using LEB as a selective enrichment broth for the isolation of *L.monocytogenes* from a food sample may result in this organism not being recovered if significant levels of *L.innocua* (i.e equal levels of *L.monocytogenes*) are also present. The IDF

(Anon, 1990) also recommends the selection of 5 *Listeria* typical colonies for identification. From this study, it was shown that when 8 isolates identified from an LEB inoculated with equal numbers of *L.monocytogenes* and *L.innocua*, all isolates were shown to be *L.innocua*. If the IDF continue to recommend LEB for detection of *L.monocytogenes*, it would be necessary to increase the number of identifications to greater than 8 to allow *L.monocytogenes* to be identified if present in the original sample. This would be both tedious and prohibitively expensive in the identification work necessary. Rather, from the results of this study, it would seem better that LRB should be recommended for detection of *L.monocytogenes* in milk and milk products by enrichment procedures rather than LEB.

Using impedance technology, it was shown that some strains of *P.roqueforti*, *P.camemberti* and *Lactococcus lactis* had an inhibitory effect on the growth of *Listeria* spp. Ryser and Marth (1988) showed that a *P.camemberti* mould used in whey cheese manufacture increased the generation time of *L.monocytogenes* in this whey when compared to uncultured cheese whey. So far this is the only report on the influence of cheese ripening moulds on the growth of *L.monocytogenes*. Others have reported that starter culture metabolism can influence the growth of *L.monocytogenes in vitro* (Raccach *et al.*, 1989; Sulzer and Busse, 1991 ; Wenzel and Marth, 1991). In this thesis, a single strain of *Lactococcus lactis* (strain E 9275) from a mixed starter culture was shown to have an inhibitory effect on the growth of *Listeria* spp. including *L.monocytogenes*. Sulzer and Busse (1991) also showed that a single strain of

Lactococcus lactis had this effect but in combination with a commercial mixed starter culture had no effect on the growth of *L.monocytogenes.*

It is unknown whether the effect of the *Lactococcus lactis* strain E 9275 against *L.monocytogenes* used in this study would be lost if it was used in a mixed starter culture or if this single *Lactococcus lactis* strain could be used as a sole starter culture in cheese manufacture. Future work would need to be carried out to answer these questions. This work would involve growth of the *Lactococcus lactis* strain E 9275 with other *Lactococcus lactis* strains. These strains could be grown together in TSB and spent broth supernatant collected as before (see Chapter 4). Using impedance technology, this broth could be compared with the growth of *L.monocytogenes* in control TSB as before (see Chapter 4). To examine the possibility of using *Lactococcus lactis* strain E 9275 as a sole starter culture in cheese manufacture, this could be done by manufacturing a cheese using this starter culture and examining how effective it was at lowering the pH of the milk to allow the milk to coagulate and produce a cheese curd.

These results indicate that selection of strains of fungal cheese ripening moulds which are inhibitory for *Listeria* could affect the overall growth of *Listeria* spp. in cheese, resulting in possible bacteriologically safer mould-ripened cheese. Further work would need to be carried out on these *Listeria* inhibitor cultures to make sure that cheese manufactured using these cultures would produce an organoleptically acceptable cheese with the correct flavour and texture. Future work would involve manufacturing cheese using these *Listeria* inhibitory cheese ripening moulds and analysing the cheese

composition for total solids, moisture, fat, crude protein, pH, salt, fat in dry matter, moisture in non-fat solids, salt in moisture to show if a desirable cheese was produced. Correct flavour and texture could be show by sensory analysis involving taste panels. Similar work involving cheese composition and sensory analysis has been done previously at the Hannah Research Institute with other cheese types.

The strains of fungal cheese ripening moulds inhibitory for *Listeria* were used to produce semi-soft mould ripened cheese. The results from these experiments showed that the *in vitro* effects could generally not be shown in the manufactured cheese. However, in an experiment with a Camembert cheese ripened for two weeks then inoculated with *L.monocytogenes* on the outside to stimulate post-manufacture contamination, it was shown that *P.camamberti* strain Niege previously shown to be inhibitory *in vitro* was also shown to be inhibitory to *L.monocytogenes* on the surface of the cheese. Contact between the mature fungal growth and the *L.monocytogenes* may account for the detectable levels of inhibition of *L.monocytogenes* in a final cheese product. Future work to prove this inhibitory effect on *L.monocytogenes* with mature fungal growth would involve manufacturing mould ripened cheese using the *L.monocytogenes* inhibitory fungal strains. Once the mould growth was apparent on (Camembert cheese) or in (Blue-veined cheese), then the cheese could be inoculated with *L.monocytogenes.* This could then be compared with other cheese fungal strains shown previously to have no effect on the growth of *L.monocytogenes in vitro* (see Chapter 4).

From these experiments, the survival and growth of *L.monocytogenes* in soft and semi-soft cheese (regardless of fungal strain used) was shown. It was shown that *L.monocytogenes* could not survive in Blue-veined cheese made from raw milk containing *L.monocytogenes.* This was also shown by Papageorgious and Marth (1989). Salt content (in the range of 3-5%) and moisture (40-50%) are particularly high in blue-veined cheese and will result in a high brine concertration in the cheese. All these factors will produce an unfavourable environment for the growth of *L.monocytogenes.* No surface contamination experiments with Blue-veined cheese and survival of *L.monocytogenes* were carried out during this study. It has recently been shown that a sheep milk cheese manufactured using the same method employed in this study was found to be contaminated with *L.monocytogenes.* Since this thesis showed that *L.monocytogenes* could not survive in this cheese when inoculated into the raw milk, it would seem to suggest that contamination could only have resulted from post-manufacture surface contamination.

However, it was shown that *L.monocytogenes* could survive and grow in Camembert cheese manufactured using raw milk contaminated with *L.monocytogenes.* Even at low inoculum levels of 1 and 10 cfu/ml of *L.monocytogenes* in milk, the resulting cheese had high levels of *L.monocytogenes* even after 14 days of ripening at which time it can be sold to the public. This type of cheese can be ripened for as long as 5 weeks after manufacture at refrigeration temperatures. It was shown from this study that *L.monocytogenes* continued to grow in Camembert cheese throughout this time to reach levels between 10^4-10^5 cfu/g. This study has shown that Camembert cheese should

only be manufactured using milk which does not contain *L.monocytogenes.*

Since *L.monocytogenes* does not appear to survive High Temperature Short Time (HTST) pasteurisation (71.7°C 15s) in cow milk (Bradshaw *et al.* 1985; Beckers *et al.* 1987; Lovett *et al.* 1990), this measure could be a means of destroying this organism if present in raw milk in order to produce Camembert cheese which is free of *L.monocytogenes.*

Sheep milk was found to have factors which protected Gram-negative and Gram-positive bacteria against heating (Chapter 6). Protective factor(s) were shown in sheep skim milk and sheep milk fat. This protective effect was not shown with cow or goat milk even when it was artificially made up to 5 and 10% homologous fat content. These higher fat levels were selected as sheep milk is generally of a higher fat content (5.8-9.1%) than cow (3.2-5.1%) or goat (2.8-6.5%) milk (Tamine *et al.*, 1991). It was also shown that no differences in the susceptibility to heat treatments could be shown between non-pathogenic *L.innocua* and pathogenic *L.monocytogenes.* At this time, the protective factor(s) shown in sheep skim milk and sheep milk fat is unknown. Future work would be necessary to identify this factor(s). This could be done by comparing the components of sheep milk with those of cow and goat milk. Any differences could then be isolated and their effect on *L.monocytogenes* during heating at 65°C could be determined. Influence of these factor(s) on other pathogens should also be studied.

The Milk (Special Designation) Regulations 1989 state that for pasteurised whole cow milk the heat treatment should be not less than 62.8°C

and not more than 65.6°C for at least 30 min (batch pasteurisation) or not less than 71.7°C for at least 15 s (plate pasteurisation) or an equivalent process. Batch pasteurisation is normally carried out by small cheese and dairy product manufacturers using a cheese vat. These are similar in design to water baths but with the water being replaced by milk. The experiments for batch pasteurisation were carried out in test tubes heated in a water bath. It was shown that the psychrotrophic Gram-negative isolates in all milk types and *Listeria* spp. in cow milk (5% fat) could not survive batch pasteurisation while *Listeria* spp. in sheep milk (0-10% fat) could survive batch pasteurisation.

It was shown that by increasing the batch pasteurisation to 68 and 72°C, *Listeria* spp. could not survive at 68°C after 45 min and at 72°C after 15 min in whole sheep milk. Gram-negative isolates could survive batch pasteurisation at 72°C after 15 min but not 30 min in whole sheep milk. At this present time, there is no legislation governing the pasteurisation heat treatment for sheep milk. In considering the results of this study, it would be advisable to pasteurise whole sheep milk at 72°C for 30 min with the batch pasteurisation method to ensure destruction of Gram-negative psychrotrophic organisms and *L.monocytogenes.*

It was shown that high levels of *L.monocytogenes* ($1x10^6$ cfu/ml) contained in whole sheep, cow and goat milks could not survive the current HTST plate pasteurisation protocol.

In Europe, the process of thermisation is used to treat raw milk in order to extend cold-storage of the milk. The IDF (Anon, 1981) describes thermisation as "heating to 63-65°C, holding for 15-20 s and then cooling,

with the result that psychrotrophic bacteria are almost destroyed while for the greater part the milk enzymes are unaffected''. The milk can then also be pasteurised before being used. In Canada a treatment at 62.8°C or 65.6°C is used for thermisation of milk intended for long-hold Canadian cheddar manufacture.

It was shown that milk inoculated with *L.monocytogenes* at a level of approximately 10^3 cfu/ml which was then thermised at 62.8°C and 65.6°C showed detectable levels of *L.monocytogenes* by direct plating or by enrichment culture. Mackey and Bratchell (1989) have also shown that *L.monocytogenes* would survive in raw milk thermised between 60 and 65°C for 16 s.

It was shown that long-hold Canadian cheddar and Camembert cheese manufactured using thermised milk at 62.8°C and 65.6°C previously inoculated with 10^3 cfu/ml *L.monocytogenes* was shown to contain *L.monocytogenes* at the end of the ripening period. It was shown that thermisation was not a suitable heat treatment for removing pathogens from these cheese types as the final product still contained *L.monocytogenes.* Milk intended for these purposes would still require proper pasteurisation protocols before manufacture.

It was however shown that thermisation was a suitable heat treatment for milk used to manufacture Danish blue cheese. It should be noted that raw milk inoculated with 10^3 cfu/ml *L.monocytogenes* and used to manufacture this cheese was shown to contain no *L.monocytogenes* after 11 weeks of ripening. It has been shown that this cheese type can be manufactured using raw milk containing *L.monocytogenes* and still be free of *L.monocytogenes* in the final

ripened product.

It was also found that thermisation does not compromise subsequent HTST pastuerisation by inducing tolerance to pasteurisation in *L.monocytogenes*, so this technique can safely be used to reduce the milk flora in the interim storage period before pasteurisation.

In summary, it can be concluded that sheep milk contains a heat protective factor(s) which allows increased heat resistance for *L.monocytogenes*. In all the milk types studied, *L.monocytogenes* does not survive HTST pasteurisation. This organism can survive readily in Camembert cheese and long-hold Canadian cheese even in milk given a prior heat treatment of between 62.8-65.6°C for 16 s. *L.monocytogenes* does not survive in Blue-veined and Danish blue cheese manufactured using raw milk inoculated with this organism. This study has confirmed that some raw milk and cheese samples purchased in Scotland contained *L.monocytogenes* and it could be concluded that this type of product poses a potential risk of carrying *L.monocytogenes*.

For the dairy industry as a whole, the isolation and identification of *L.innocua* from their products should be regarded with caution. Either they should increase the number of presumptive *Listeria* isolates identififed to increase the likelihood of identifying *L.monocytogenes* or make the assumption that *L.monocytogenes* is present in their product due to selective nature of the *Listeria* enrichment broths for *L.innocua* they are using and act accordingly. The time and cost to impliment these changes would make it very unlikely that the dairy industry would comply. An alternative at this time would be to

introduce new methods where *L.monocytogenes* was being specifically targetted such as *L.monocytogenes* specific monoclonal antibodies. This would allow the dairy industry to aware of any problems in their products which current methods do not highlight and which could result in products being on the market which contain *L.monocytogenes* and that could be consumed by all sections of the population.

Since the 1989-1990 outbreaks of human listeriosis in the U.K. have dropped markedly. It would seem that the warning given by the Department of Health advising pregnant woman and immunocompromised people to avoid eating soft cheese, to adequatly reheat cook-chilled meals (Cumber *et al*, 1991), avoid paté (Anon, 1989) and the removal of a source of *L.monocytogenes* shown to be a particular brand of paté (Gilbert *et al.*, 1993) has helped to greatly reduce the number of reported cases of listeriosis in the UK. However, worldwide outbreaks of listeriosis have continued as in the outbreak in France (Salvat *et al.*, 1995). This may therefore, at this present time occur again so the dairy industry must still continue to monitor for the presence and incidence of *Listeria* spp. in their products as well as the factory environment as an outbreak of listeriosis will result in a high mortality rate (36%) (Farber and Peterkin, 1991).

CHAPTER 10

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