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Manatū Ahu Matua

Evaluation of the microbial safety of raw milk cheeses: Investigation into the effect of milk time and temperature storage variables, together with internal mould ripening, on the survival and growth of ***Listeria monocytogenes*** during soft cheese production

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Prepared for MPI by Dr Susan Paulin (ESR and Dr Tanya Soboleva & Dr Sally Hasell (MPI)

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Scientific Interpretive Summary

This SIS is prepared by MPI risk assessors to provide context to the following report for MPI risk managers and external readers

Evaluation of the microbial safety of raw milk cheeses: Investigation into the effect of milk time and temperature storage variables, together with internal mould ripening, on the survival and growth of *Listeria monocytogenes* during soft cheese production. ESR Report FW13024

Manufacturing of raw milk products, including soft cheeses, is now allowed in New Zealand. The safety of these products requires certain factors to be controlled, including the conditions under which milk is collected and stored prior to the commencement of fermentation. To make raw milk products the milk must either be used within 2 hours of collection or it may be chilled but must still be used within a stated period. This study looked at the impact of the storage conditions on growth and survival of *Listeria monocytogenes,* the pathogen of greatest concern in the safety of raw milk products, especially soft cheeses where there is a long fermentation period. There are reports that a lag phase is induced by the chilling of the milk.

In this study, the trial conditions did not find a lag phase when a cocktail of selected strains were inoculated into milk held under the permitted conditions for the maximum permitted periods. When the experiment was repeated in a laboratory-based brie-style cheese, while there was no difference in growth rates during the first 2 hours (indicating that no lag phase had been induced by chilling) at 21 hours counts indicated increases of about 2 logs for chilled milk compared to 1 log for the cheeses made with the un-chilled milk. This effect did not appear to be due to significant differences in the rate at which the pH dropped in the two types of cheeses or the final pH at 24 hours. As no further readings were taken it is not known if this difference would have been sustained.

The second experiment conducted evaluated the impact of the presence of an internal mould on the survival of *L.monocytogenes* in cheeses made with fresh, un-chilled raw milk. Concerns have been raised as to the relative safety of these cheeses, as the pH changes induced by the mould are known to provide conditions suitable for growth of *L.monocytogenes.* While it was found that the bacteria grew to higher levels in cheeses with the added mould, especially internally, the presence of surface moulds acquired from the environment on the uninoculated cheeses, meant that they too showed bacterial growth.

These studies provide different outcomes to other reports, although these are limited. With the *L.monocytogenes* strains used in this study, no lag phase was found to be induced by the storage conditions and growth of the pathogen was found during the initial fermentation. While this was a small study, the results do indicate that *L.monocytogenes* can grow during the manufacture and ripening of *brie-style* soft cheeses and when internal mould ripening also occurs, the growth is enhanced. This means a small number of *L.monocytogenes* present in the raw milk could increase during the manufacturing process, meaning that this type of raw milk product would not be permitted by the current regulations.

The study suggests that rather than chilling milk prior to the commencement of cheese-making inhibiting pathogen growth, un-chilled milk may have some inhibitory effects.

Client Report

FW13024

Evaluation of the microbial safety of raw milk cheeses:

Investigation into the effect of milk time and temperature storage variables, together with internal mould ripening, on the survival and growth of *Listeria monocytogenes* during soft cheese production.

Dr Stephen On

Chief Scientist, Environmental Science

Dr Susan Paulin

Project leader

Dr Beverley Horn

Mr Maurice Wilson

Peer reviewers

Evaluation of the microbial safety of raw milk cheeses:

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By

Dr Susan Paulin

September 2015

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TABLE OF CONTENTS

INDEX OF TABLES AND FIGURES

SUMMARY

This study was conducted to determine whether the currently-permitted cooling and storage conditions of raw milk, intended for making raw milk cheese, had an effect on the growth and survival of *L. monocytogenes* during the manufacture of soft Brie-like cheeses. Secondly, this work aimed to evaluate whether the growth of *L. monocytogenes* during ripening was influenced by the addition of *P. roqueforti* which is commonly used for internal mould ripening.

At the beginning of this study growth curve experiments were undertaken to validate the suitability of the selected *L. monocytogenes* isolates for this study. During the 8 hours of observation all five isolates grew at 37°C in both raw and pasteurised milk, at both high (5 $\log_{10}$ CFU/ml) and low (3 $\log_{10}$ CFU/ml) starting bacterial concentrations. It was also shown that chilling inoculated milk and then rapidly warming it up did not change the growth behaviour the *L. monocytogenes* isolates. These results showed the isolate cocktail was suitable for use in the required cheese making experiments.

In the first experiment, the initial 24 hours of the manufacturing process of soft Brie-like cheese was replicated in the laboratory. *L. monocytogenes* was inoculated into the fresh raw milk, which was then divided in two parts: one was used immediately for cheese making (referred to throughout as un-chilled milk) and another was chilled for 24 hours at 4°C prior to the start of cheese making (referred to throughout as chilled milk). The concentration of *L monocytogenes* was determined at 0 hours, 2 hours, 6 hours and then three hourly until 24 hours after the start of cheese manufacture. *L. monocytogenes* concentrations were expressed on a dry weight basis to allow comparison of various stages of cheese manufacture.

The experiments showed growth of *L. monocytogenes* during the early stages of raw milk soft Brie-like cheese manufacture. The growth rate of *L. monocytogenes* for the first two hours of manufacture was the same for cheeses using the chilled and un-chilled milk. Temperature shock due to the fast chilling to 4°C and then, after 24 hours, rapid warming up to 32°C did not lead to a significant delay in the growth of *L. monocytogenes.*

At 21 hours, a 1 $\log_{10}$ CFU/g higher concentration of *L. monocytogenes* was observed in the cheeses made with chilled milk compared to the cheeses made with un-chilled milk. However,

it is not possible to say if this extra growth would be sustained beyond 24 hours when the experiment concluded.

This study showed that the growth of *L. monocytogenes* in cheeses made with chilled milk can be equivalent or greater to that in cheeses made with un-chilled milk used shortly after milking. Delayed growth of *L. monocytogenes* observed in some experiments previously reported in the literature might be specific to the particular raw milk type and *L. monocytogenes* isolates used in those experiments.

In the second set of experiments fresh (within 2 hours of milking) raw milk was used to manufacture soft Brie-like cheeses with or without the addition of *P. roqueforti*. These cheeses were allowed to ripen for 42 days at 13°C in a high humidity atmosphere. *L. monocytogenes* was inoculated into the milk prior to the start of cheese manufacture and the concentration of *L. monocytogenes* was measured weekly during ripening.

During ripening *L. monocytogenes* was able to survive and then grow inside and on the surface of soft raw milk cheeses manufactured with or without *P. roqueforti*. For all cheeses the growth of *L. monocytogenes* was more rapid on the exterior, compared with the interior of the cheeses. This could be explained by the presence of air-borne surface mould growth which naturally colonises the exterior surfaces of the cheeses and by doing so increases the pH of the cheese surface.

In these experiments the presence of *P. roqueforti* in the soft cheeses resulted in increased growth of *L. monocytogenes*, compared to the same cheeses without added *P. roqueforti*.

In the *P. roqueforti* cheeses, an increase in *L. monocytogenes* concentration in the early stages during ripening was observed. Bacterial growth started between days 14 and 21 inside the *P. roqueforti* cheeses, compared to between days 21 and 28 days inside cheeses with no *P. roqueforti*. On the surface of the cheeses, initial growth was observed between 7 and 14 days for *P. roqueforti* cheeses, and between 14 and 21 days for cheeses with no *P. roqueforti*. These increases in *L. monocytogenes* concentration corresponded to increases in the pH of the cheese. The pH of the cheese increased due to actively growing mould catabolising lactic acid to carbon dioxide and excreting enzymes to produce ammonia.

An increased maximum sustained concentration of *L. monocytogenes* during ripening was also observed inside cheeses made with added *P. roqueforti* (8.5 to 8.9 $\log_{10}$ CFU/g) compared to cheeses with no added *P. roqueforti* (5.5 to 6.5 $\log_{10}$ CFU/g).

The difference in growth behaviour of *L. monocytogenes* in the two cheese types is mostly due to the changes in pH. The growth of *L. monocytogenes* inside the *P. roqueforti* cheeses may be inhibited by fatty acids produced as the *P. roqueforti* mould starts to grow. The fatty acids are themselves broken down during cheese ripening, so only provide a transient inhibition of *L. monocytogenes.* Increases in the concentration of *L. monocytogenes* are subsequently likely to occur once the environment within the cheese becomes more amenable to bacterial growth.

1 INTRODUCTION

Milk and milk products provide a rich source of nutrients that can support the survival and growth of many bacterial species. While heat treatment of raw milk (e.g. pasteurisation or thermisation) is an effective method of controlling pathogens without compromising product quality, there is a growing consumer demand for untreated (or raw) milk and milk products, including cheese. During the early stages of cheese manufacture, conditions including temperature and pH are close to optimal for supporting the growth of pathogens. Consequently, if pathogenic bacteria such as *Listeria monocytogenes* are present in raw milk prior to cheese making, the raw milk cheese may represent a risk to the consumer.

As the cheese making process progresses to the ripening or maturation stages, the environment within the cheese can change due to many factors, including availability of salt or moisture, alterations in the pH, the presence of other microorganisms (including moulds such as *Penicillium roqueforti* which may be added for flavour and texture enhancement) and the microflora naturally present in raw milk. The complex physico-chemical changes that occur during the cheese making process and subsequent ripening can influence the rate of pathogen growth and survival in the product.

L. monocytogenes is an important foodborne pathogen (Nightingale *et al*., 2004, Wiedmann and Evans 2011). Dairy products, including raw milk and cheese, are well-recognised routes for the transmission of *L. monocytogenes* to humans (Harvey and Gilmour 1992, Gaya *et al*., 1996). There have been many reports of outbreaks associated with the consumption of such products. These data have been extensively reviewed recently by King *et al*., 2014 and Paulin *et al*., 2014.

L. monocytogenes can survive and grow at refrigeration temperatures (Lianou and Koutsoumanis 2013) which could increase the risk to human health from raw milk stored prior to processing. There are strict regulations in place within New Zealand covering the production and processing of raw milk products[1]. Unless further processing commences within two hours from the completion of milking, the milk must be immediately cooled as described in NZCP1: Code of

[1] http://www.foodsafety.govt.nz/elibrary/industry/raw-milk-products-cop/code-of-practice-additional-measures-for-raw-milk-products.pdf (Accessed 11th September 2015)

Practice for design and operation of farm dairies[2]. The information regarding milk cooling is summarised as follows:

a) Raw milk must be cooled to 7°C or below within three hours of milking if the milk is stored for no more than 24 hours.
b) Raw milk must be cooled to 6°C or below within two hours from the completion of milking.

Raw milk held at 6°C or below must be used for further processing within 48 hours.

In a recent New Zealand study, milk was held at 4°C for up to 24 hours prior to the addition of *L.monocytogenes* and starter culture. Milk cultures were then incubated at 30ºC for the duration of the sampling period. *L. monocytogenes* did not grow for up to 12 hours after inoculation despite conditions being ideal for growth (Withers and Couper, 2012). Another study has obtained similar results demonstrating that *L. monocytogenes* did not grow in raw milk for 3-5 days when held at 4°C (Farber *et al*., 1990). It has been suggested that this could be due to lag phase induced by exposure to chilling temperatures prior to rapid warming at the start of cheese making. As milk does not have to be chilled if the cheese making begins within two hours of the end of milking (Code of Practice: Additional measures for raw milk products, 2010) bacteria held at a suitable temperature for growth may be able to grow more rapidly, in the absence of a lag phase, particularly during the early stages of cheese making. Alternatively, actively-growing *Listeria* could be more susceptible than lag phase bacteria to inhibition by the increasing acidity or to other pathogen inhibitory factors that are naturally present in raw milk at this time (Pitt *et al*., 1999).

The first objective of this study was to determine whether the currently accepted time and temperature variables associated with cooling and storage of raw milk, prior to the start of cheese making, had an effect on the growth and survival of *L. monocytogenes* following manufacture of soft, Brie-like cheeses. A young, soft cheese was selected for laboratory manufacture in this study as traditionally the process of making such cheeses includes a long fermentation period

[2] http://www.foodsafety.govt.nz/elibrary/industry/dairy-nzcp1-design-code-of-practice/amdt-2.pdf (Accessed 11th September 2015)

where conditions are highly favourable for bacterial growth. Furthermore, cases of *L. monocytogenes* foodborne illness, associated with cheese consumption are more commonly, although not exclusively, attributed to soft young cheeses rather than aged harder varieties (Paulin *et al*., 2014).

The addition of blue mould at the start of cheese manufacture and its subsequent growth is known to have an effect on the pH of cheeses and the production of fatty acids during ripening, which has been suggested to influence the survival of *L. monocytogenes* (Papageorgiou and Marth, 1988). The second objective of this study was therefore to determine whether internal mould ripening, following the addition of *P. roqueforti* at the start of cheese manufacture, influenced the growth and survival of *L. monocytogenes* in soft Brie-like cheeses made from raw, un-chilled milk.

The following two experiments were conducted to address the above objectives:

1) Cheese manufacture:
- ↗ Using raw, un-chilled, fresh milk
- ↘ Using raw chilled (for 24 hours) fresh milk

2) Cheese manufacture and ripening using raw, un-chilled fresh milk:
- ↗ Plus *Penicillium roqueforti*
- ↘ No *Penicillium roqueforti*

2 MATERIALS AND METHODS

The methods described in the following sections detail the procedures used for screening and selecting *L. monocytogenes* isolates, pre-cheese making, *in vitro* isolate characterisation and investigation of the survival and growth of *L. monocytogenes* during soft Brie-like cheese manufacture and internal mould ripening under laboratory conditions.

2.1 Milk source

A local farm, within 15 km of ESR's Christchurch Science centre was selected to supply all of the raw milk for the isolate characterisation and cheese making experiments. The period of milk collection was between February and May 2014. For pathogen growth experiments that required pasteurised milk, non-homogenised Farmhouse milk (MeadowFresh) was used.

2.1.1 Milk cooling

According to the Code of Practice: Additional measures for raw milk products (2010) http://www.foodsafety.govt.nz/elibrary/industry/raw-milk-products-cop/code-of-practice-additional-measures-for-raw-milk-products.pdf, unless cheese manufacture commences within two hours from the completion of milking, milk must be cooled to 7ºC or below within three hours and the milk stored for no more than 24 hours prior to use. In order to ensure that these requirements are met, optimisation of milk cooling was undertaken. Milk arriving at ESR was immediately filtered, measured into a plastic container (7 litres) and transferred to a walk in freezer (-20ºC). Every 30 minutes, the milk was agitated, to ensure good mixing, and the temperature profile recorded. Milk reached a temperature of approximately 4ºC within three hours. Once this temperature was reached, the milk was transferred into a refrigeration room and left for 24 hours prior to use for the cheese making experiments.

2.2 Selection and screening of *L. monocytogenes* isolates

Extensive enquiries were made in order to obtain *L. monocytogenes* isolates originating from a variety of different sources (and different serotypes) including those from dairy and cheese environments. Eight isolates were obtained from the ESR New Zealand Reference Culture Collection, Medical Section (NZRM) (Table 2). These were screened and, based on available isolate information, morphological and physical characteristics (excluding any isolates that were slow growers or exhibited atypical colony morphology), five isolates were selected, which were

used individually and as a cocktail for the growth curve experiments and as a cocktail for the cheese making trials.

2.2.1 Agar selection

Isolates were each grown on three types of agar selective for *L. monocytogenes* (PALCAM, ALOA and Oxford (*Listeria* selective agar)) in order to determine the most appropriate medium to use in the trial. All isolates grew best on ALOA agar plates with any non-*L. monocytogenes* colonies present in the milk being easily identifiable. This medium was therefore selected for use throughout the trial.

2.2.2 Microgen, CAMP *Listeria* identification test, PFGE confirmation and isolate inhibition tests

All isolates were tested for *Listeria* spp. identification using the Microgen® *Listeria* ID kit, the CAMP *Listeria* identification test (Groves and Welshimer, 1977) and PFGE analysis (using the restriction enzymes *Asc*I and *Apa*I). Furthermore, all isolates were tested for non-inhibition to the normal growth of each of the other isolates using a streak-plate antagonistic assay.

2.3 Growth curve characterisation of selected *L. monocytogenes* isolates

Five individual *L. monocytogenes* isolates, and the cocktail of isolates, were characterised prior to the cheese making experiments by undertaking a series of *in vitro* growth experiments. Growth curves: raw and pasteurised milk

2.3.1 Growth curves: raw and pasteurised milk

L. monocytogenes isolates were streaked for single colonies on ALOA agar plates and inoculated into Tryptic Soy broth (TSB) at 37°C for 24 hours. Bacteria were sub-cultured into fresh, pre-warmed milk (pasteurised and raw) to obtain a starting inoculum of either 3.0 $\log_{10}$ CFU/ml or 5.0 $\log_{10}$ CFU/ml. All growth curves for these experiments were performed at 37°C and samples were taken in duplicate at each time point. Samples were taken at six time points: T0 (immediately after sub-culturing into milk), T2hr, T4hr, T6hr, T8hr and T24hr and were diluted in phosphate buffered saline (PBS) (Fort Richard Laboratories, Auckland, NZ) before plating onto ALOA agar. Plates were incubated at 37°C for 24 hours and *L. monocytogenes* colonies enumerated.

2.3.2 Raw milk growth curves: un-chilled and chilled milk

L. monocytogenes isolates were streaked for single colonies on ALOA agar plates and inoculated into Tryptic Soy broth (TSB) at 37°C for 24 hours. Bacteria were sub-cultured into fresh, pre-warmed, raw milk to obtain a starting inoculum of 3.0 $\log_{10}$ CFU/ml. Half of the milk was used for growth curve analyses immediately and the other half was chilled to 4°C and held for 24 hours prior to warming to 32°C and commencement of the growth curve analyses. All growth curves for these experiments were performed at 32°C and samples were taken in duplicate at each time point. Samples were taken at six time points: T0 (immediately after sub-culturing into milk), T2hr, T4hr, T6hr, T8hr and T24hr and were diluted in phosphate buffered saline (PBS) (Fort Richard Laboratories, Auckland, NZ) before plating onto ALOA agar. Plates were incubated at 37°C for 24 hours and *L. monocytogenes* colonies enumerated.

2.4 Laboratory manufacture of raw milk soft Brie-like cheeses infected with *L. monocytogenes* (Experiment 1)

2.4.1 Treatment of raw milk

Raw milk was transported from the farm to the laboratory in sterile, plastic 20L Bio-waste containers with sealable lids. As soon as the milk arrived at the laboratory, it was filtered through sterile dairy sleeves, to mimic the double filtration applied at the farm, and a sample plated onto ALOA plates to ensure that no *L. monocytogenes* was present in the milk. Seven litre volumes were either transferred into sterile 13L pots (within 17L pots acting as a double boiler), and equilibrated to 32°C ready for immediate cheese manufacture (**Un-chilled milk, Day 1**) or the milk was chilled (as described in Section 3.1.1) to be used for cheese manufacture the following day (**Chilled milk, Day 2**). Prior to the commencement of cheese manufacture on Day 2, the chilled milk was warmed quickly to 32°C (using a double boiler and hot plate). The experimental procedure used is diagrammatised in Appendix 1.

2.4.2 *L. monocytogenes* inoculation of raw milk

The five *L. monocytogenes* isolates selected for the experimental work were grown individually overnight in TSB at 37°C. Isolates were diluted and combined to achieve a cocktail containing a final *L. monocytogenes* concentration of approximately 3.0 $\log_{10}$ CFU/ml. Bacteria were added to the raw milk (with the exception of the un-inoculated controls) immediately after filtration

and distribution into 7L volumes. The inoculum was gently stirred through the raw milk to achieve even distribution.

2.4.3 Cheese manufacture (Experiment 1)

The cheese making method used was adapted from Neil Willman (Willman and Willman, 2005) and is similar to that used in a previous experimental trial (Paulin, 2013). The laboratory-based manufacturing procedure is summarised in Table 1.

A total of nine cheeses were manufactured, from raw milk, on two separate days:

- 4 cheeses on **Day 1** (using un-chilled, raw milk): Three 'test' cheeses: C1, C2 and C3 (inoculated with an *L. monocytogenes* cocktail) and one 'control' cheese: C4 (no added *L. monocytogenes*).
- 5 cheeses on **Day 2** (using chilled raw milk): Three 'test' cheeses: C5, C6 and C7 (inoculated with an *L. monocytogenes* cocktail) and two 'control' cheeses: C8 and C9 (no added *L. monocytogenes*). Cheese 9 was used for a practice ripening trial in order to verify both the cheese cave conditions and the post manufacture handling for reduction of mould growth.

Table 1. Description of the ESR laboratory-based manufacture of raw milk soft Brie-like cheeses.

Activity	Description of activity
Milk	Raw milk was treated as described in Section 3.4.1.
L. monocytogenes cocktail	*L. monocytogenes* isolates (cocktail of 5 isolates: (LM101, LM1, NZRM 3450, NZRM 3370 and NZRM 3371) at 3.0 $\log_{10}$ CFU/ml (final concentration) were added to the raw, un-chilled milk (not control cheeses) as described in Section 3.4.2.
Add starter culture and mould spores (**Time 0hr**)	FD-DVS Flora Danica starter culture (50U/1000L) and *P. roqueforti* (10U/1000L) blue mould spore powder was added to all of the warm milk 7L volumes. Milk was mixed gently and fermented for 90 minutes at 32ºC. NB: *P. roqueforti* was added to some of the Experiment 2 cheeses only.
Add rennet	Rennet was added to the milk (0.16 g diluted in 1.6 ml cooled boiled water per cheese) and mixed well for 1-3 minutes. The temperature was maintained at 32ºC and the cheeses left to set for an additional 40 minutes.
Cut curds	The temperature was maintained at 32ºC and the curd cut into cubes (approx. 2 cm).
Stop cutting curds	The curds were allowed to sit for 5 minutes.
Turn curds	The curds were gently turned three times at 10 minute intervals while maintaining the temperature at 32ºC.
Drain whey	Approximately one third of whey (2L) was removed and replaced with 2L litre of sterilised water at 35ºC. The curds were gently turned.
Hooping	Half of the whey was removed and the remaining curds and whey poured into 20 cm diameter Brie hoops lined with cheese cloth. These were placed on a wire rack over a Bio-waste container (to contain the infected whey).
Invert hoops	Hoops were carefully inverted after 10 minutes, 30 minutes, 3 hours, 6 hours, and 9 hours and then left overnight at room temperature.
pH monitoring	pH readings were taken every 30 minutes from the start (T0) until T7hr and hourly until T9hr. Thereafter pH readings were conducted every 3 hours in line with the microbiology sampling points (until T24hr). A micro-electrode 96mm spear-tipped pH probe was used for sampling (Global Science) to facilitate ease of internal measurement collection.

Microbiological samples	Samples, either 10 ml or 10 g (x3 for microbiology/x1 for dry weight analysis), were taken every three hours during the manufacture and hooping stages (up to 24 hours). An apple corer was used to vertically sample through the pressed curds. Analyses included viable counts and dry weight calculations. Samples for viable counts of *L. monocytogenes* were diluted in PBS and spread, in triplicate, onto ALOA agar plates. Plates were incubated at 37°C for up to 24 hours and *L. monocytogenes* colonies counted. Viable counts of *L. monocytogenes* were also conducted on the curds and whey that were removed following coagulation.

NB: this is the protocol for cheese manufacture only (Experiment 1). Any differences applied during cheese ripening (Experiment 2) will be discussed in the appropriate sections below.

2.5 Laboratory manufacture and ripening of raw milk soft Brie-like cheeses infected with *L. monocytogenes* (Experiment 2)

2.5.1 Treatment of raw milk

For the cheese ripening experiments, the raw milk was used filtered and un-chilled (within 2 hours from the completion of milking) as described in Section 3.4.1.

2.5.2 *L. monocytogenes* inoculation of raw milk

A cocktail of *L. monocytogenes* isolates were prepared and added to the warm milk as described in Section 3.4.2.

2.5.3 Cheese manufacture

The cheese manufacturing method used for the ripening experiments was similar to that summarised in Table 1 (and presented in Appendix 1) with the following modifications.

A total of six cheeses were manufactured and then ripened either with or without the addition of *P. roqueforti* blue mould and *L. monocytogenes*:

- Three 'test' cheeses: C1, C2 and C3 (*L. monocytogenes* cocktail **AND** *P. roqueforti*).
- Two 'test' cheeses: C4 and C5 (*L. monocytogenes* cocktail **NO** *P. roqueforti*).
- One 'control' cheese: C6 (**NO** *L. monocytogenes* cocktail or *P. roqueforti*).

2.5.4 Brining

Twenty four hours after manufacture, all cheeses (in Experiment 2) were placed in a 20% brine solution for 2.5 hours with turning after 1.25 hours. To make the brine solution, 1 kg of non-iodised salt was added to 5 litres of boiling water (per cheese) and stirred. The salt solution was cooled, 5ml white vinegar added and the solution autoclaved. Individual sterile buckets, with lids, were used for brining the cheeses. Following brining, holes were pierced in the cheeses containing the blue mould (C1, C2 and C3), using a sterile 1 ml transfer pipette, a process called 'punching' or 'needling'. This process allows oxygen to enter the interior of the cheese which is a necessary requisite for mould growth. All cheeses were then dried at room temperature, on wire racks (for several hours), before being moved to the cheese ripening room.

An experimental cheese ripening room was set up and optimised to provide suitable temperature (13°C) and relative humidity (85-90%) levels for maturation. An existing cool room was used for this purpose ensuring that temperature and humidity could be reproducibly controlled. Prior to use, the room was fully decontaminated (using several bleach scrubs), and a portable UV light source was connected to assist in the control of mould spores. Environmental samples (taken from the air and surfaces) in the cheese ripening room showed the room to be free of detectable mould spores and pathogenic bacteria.

2.5.5 Care of the ripening cheeses

Cheeses were ripened under optimal conditions for a period of 6 weeks from manufacture. Each set of cheeses (Cheeses 1-3; Cheeses 4-5 and Cheese 6) was placed on racks within individual plastic containers and ripened on wooden shelves.

Following the start of ripening, cheeses were turned daily for 10 days and the surface gently washed with a weak (2%) salt solution to help prevent external mould growth. Thereafter, the cheeses were wrapped in breathable paper and turned weekly. Each time the cheeses were removed for sampling, the UV source was activated within the room for a period of several hours to help reduce airborne moulds.

2.5.6 Cheese sampling (ripening)

2.5.6.1 pH readings

pH readings were taken approximately every hour from T0hr until T7hr, then at 10hr, 12hr and 24hr. Thereafter pH readings were taken weekly along with the microbiology and water activity sampling points. From day 21, pH readings were taken from internal and external locations for each cheese and for cheeses 1-3 (containing *P. roqueforti* mould), internal readings were also taken from areas containing mould growth (as determined via vertical sampling through the pressed cheeses), and areas free from mould growth.

2.5.6.2 Microbiological analysis

Triplicate samples were taken at T0hr, T6hr, T12hr, T24hr, Day 7, Day 14, Day 21, Day 28, Day 35 and Day 42 for microbiological analyses as described in Table 1. From 24 hours onwards,

inside and outside bacterial counts were sampled separately from each cheese. Dry weight analysis was conducted as described in section 3.6.

2.5.6.3 Water activity

For the cheese ripening experiments, an additional sample was taken at each time point for water activity determination. Measurements were calculated, using a water activity meter (AquaLab), by following the instructions in the operator's manual (Version 1.1). Samples collected were stored in screw top, plastic containers at 4°C until required.

2.6 Dry weight analysis of samples

To compare the population of *L. monocytogenes* in milk (in millilitres) and cheese (in grams) the counts were expressed as colony forming units per gram of dry weight (CFU/g dry weight). This was calculated by determining first the total solids content in cheese (IDF 1982) and milk (IDF 1987) (Schvartzman *et al*., 2010).

2.7 Literature Review

A literature review was conducted using the following search engines; Web of Science, PubMed and Science Direct. The following search terms where used:

- *Listeria monocytogenes* AND cheese
- *Listeria monocytogenes* AND raw milk
- *Listeria monocytogenes* AND blue mould
- *Listeria monocytogenes* AND roqueforti

Further literature was identified from the references of the literature located using the search engines. Information from the literature review relevant to the project aims are included in the discussion section of the document.

3 RESULTS

3.1 Milk cooling experiments

Milk was found to reach a temperature of approximately 4°C within three hours (data not shown). This is within the acceptable limits according to the Code of Practice: Additional measures for raw milk products (2010).

3.2 Selection of *L. monocytogenes* isolates

Eight *L. monocytogenes* isolates, including several from dairy and cheese environments, were obtained and characterised based on morphological and physical attributes. A final selection of five isolates was used, as a cocktail, for the cheese making experiments. Information about the isolates is summarised in Table 2.

Table 2. *Listeria monocytogenes* isolates selected for characterisation.

Isolate	Source	Serotype	Additional information
LM1010*	Feta cheese	1/2	Cheese sample
LM1008	Clinical	1/2	Case as above so not used
CMB120764	Cheesecake	unknown	Believed to be a contaminating meat isolate so not used
LM098/20	Ice cream	1/2	Very slow growing isolate so not used
LM1 (Wildtype 119A)*	Cheese	4b, 4d, 4e	Obtained from John Coventry, Melbourne
NZRM 3450*	Clinical	1/2a, PT 1967	Blood/perinatal sample. Patient had consumed smoked mussels
Scott A (NZRM 3370)*	Clinical	4b6	
V7 (NZRM 3371)*	Milk	1a1	

* Isolates used individually and in the final cocktail for all growth curve and cheese making experiments.

3.3 Isolate characterisation

All isolates were confirmed as being *L. monocytogenes* using the Microgen® *Listeria* ID kit, the CAMP *Listeria* identification test and PFGE analysis. Furthermore, all *L. monocytogenes* isolates selected were confirmed as being non-inhibitory to the normal growth of each of the other isolates using a streak-plate antagonistic assay.

3.4 Growth curve characterisation of selected *L. monocytogenes* isolates

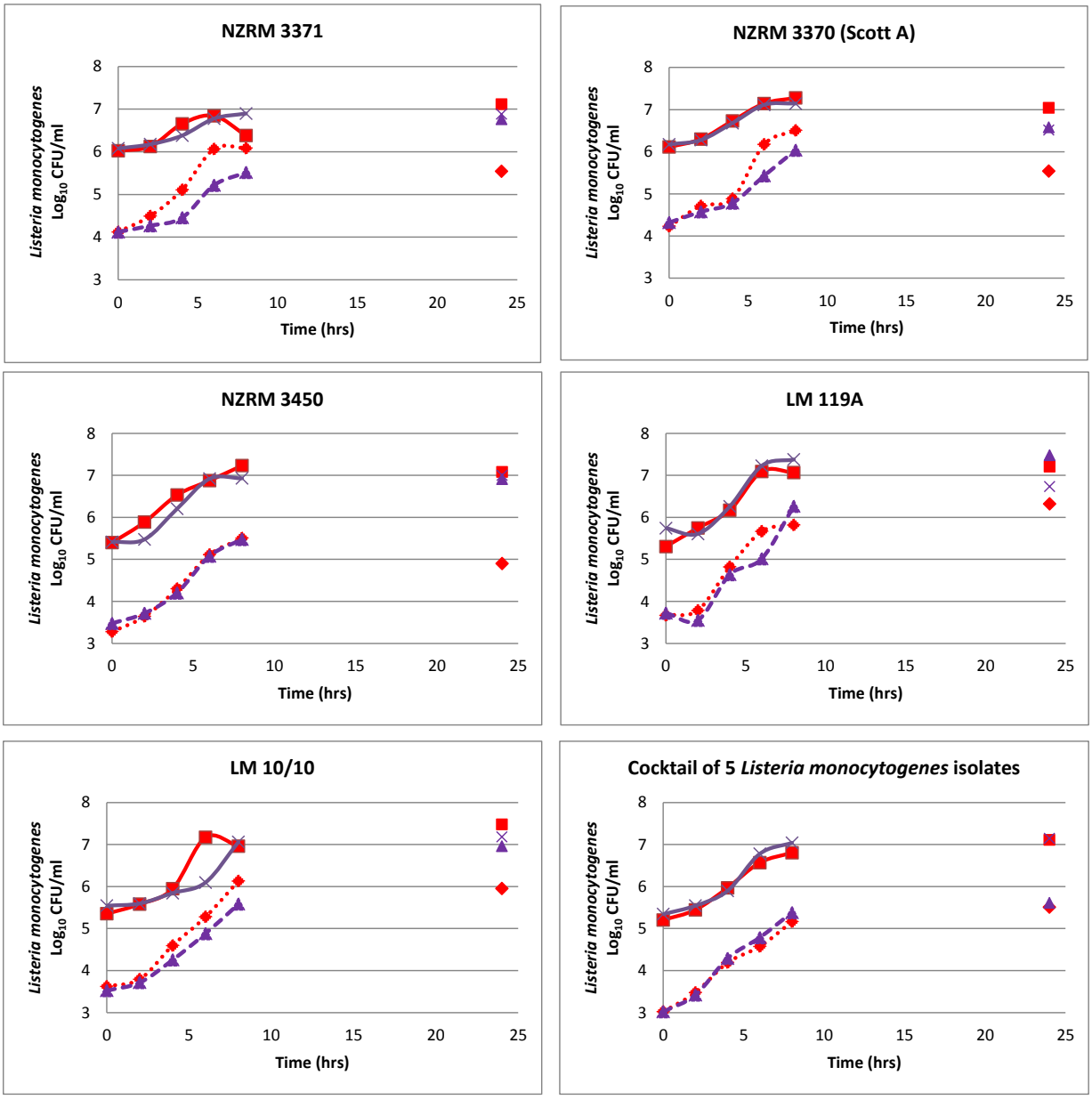
3.4.1 Growth curves using individual *L. monocytogenes* isolates and a cocktail of isolates in raw and pasteurised milk

Growth curves were conducted to determine whether *L. monocytogenes* isolates were able to survive and grow in both raw and pasteurised milk, at 37°C, using two different inoculum concentrations.

All selected individual isolates, and the *L. monocytogenes* isolate cocktail, were able to survive and grow in both raw and pasteurised milk (37°C) for up to eight hours after inoculation with starting concentrations of 3.0 $\log_{10}$ CFU/ml or 5.0 $\log_{10}$ CFU/ml. There were no differences in the growth characteristics of a cocktail of *L. monocytogenes* isolates growing in either raw or pasteurised milk (Figure 1).

The 24 hour sampling data are also shown in Figure 1. It should be noted that at the final sampling point, the milk was no longer homogeneous, thus making sampling of the milk more difficult. This may have influenced the estimated final bacterial concentration in the sample, hence making it less reliable relative to the 8 hour sample results.

Figure 1. Growth and survival of individual *L. monocytogenes* isolates and *L. monocytogenes* cocktail in raw and pasteurised milk at 37°C.



NB: Results from the cocktail growth curves are derived from two separate experiments.

3.4.2 Growth curves using individual *L. monocytogenes* isolates and a cocktail of isolates in fresh, un-chilled raw milk and raw milk that was chilled prior to the growth experiment.

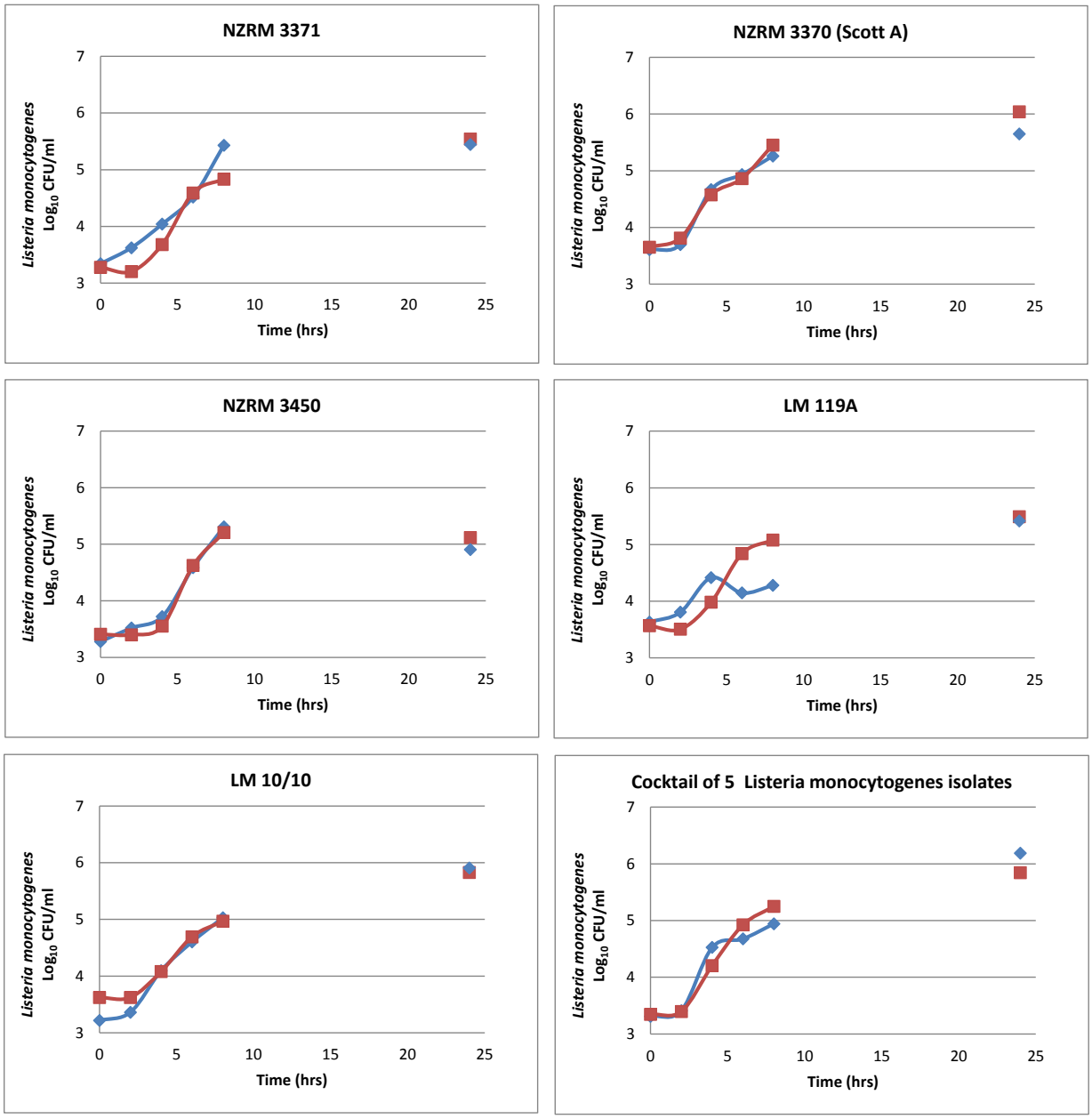
Growth curves were conducted in order to determine whether selected *L. monocytogenes* isolates, at a bacterial concentration of 3.0 $\log_{10}$ CFU/ml, were able to grow and survive when inoculated into warm, raw milk that was either used un-chilled straight after inoculation or chilled (stored at 4°C for 24 hours) prior to commencement of the growth curve experiments at 32°C.

For all isolates, including the cocktail, growth of *L. monocytogenes* was observed in the first 8 hours of growth experiments. Similar growth patterns were observed for bacteria that did and did not experience temperature shock due to chilling and sequential fast warming of milk. The maximum difference in concentration between the pathogens exposed to different temperature profiles, prior to the growth experiments, was less than 0.8$\log_{10}$ CFU/ml at any time point.

The growth experiments showed minimal growth (increase in concentration less than 0.2 $\log_{10}$ CFU/g) of all *L. monocytogenes* isolates within the first 2 hours after reaching 32°C. The only exception was strain NZRM 3371 in the un-chilled milk where after 2 hours an increase of 0.3 $\log_{10}$ CFU/g was observed. For the cocktail of 5 strains of *L. monocytogenes* the growth curves for both experimental conditions were similar with a maximum difference of 0.3 $\log_{10}$ CFU/g for samples taken at four, six and eight hours.

The 24 hour sample data are also shown in Figure 2. It should be noted that at the final sampling point, the milk was no longer homogeneous, thus making sampling of the milk more difficult. This may have influenced the estimated final bacterial concentration in the sample.

Figure 2. Growth and survival of individual *L. monocytogenes* isolates and *L. monocytogenes* cocktail in raw chilled and un-chilled milk.



NB: Results from the cocktail growth curves are derived from the average bacterial concentrations from two separate experiments with the exception of the 24 hour time point which is data derived from one experiment only.

NB: Time zero on the graph relates to the time that growth measurements commenced. For the chilled milk samples, this was inoculation plus 24 hours (i.e. chilling for 24 hours followed by warming back to 32°C).

3.5 *L. monocytogenes* growth and survival during soft Brie-like cheese manufacture (Experiment 1)

Raw milk for cheese manufacture was either used un-chilled, within 2 hours of collection at 32°C (Day 1), or was chilled to 4°C and stored for 24 hours prior to warming to 32°C before use (Day 2). A flow chart detailing experimental conditions used for the manufacture of cheeses in Experiment 1 is presented in Appendix 1.

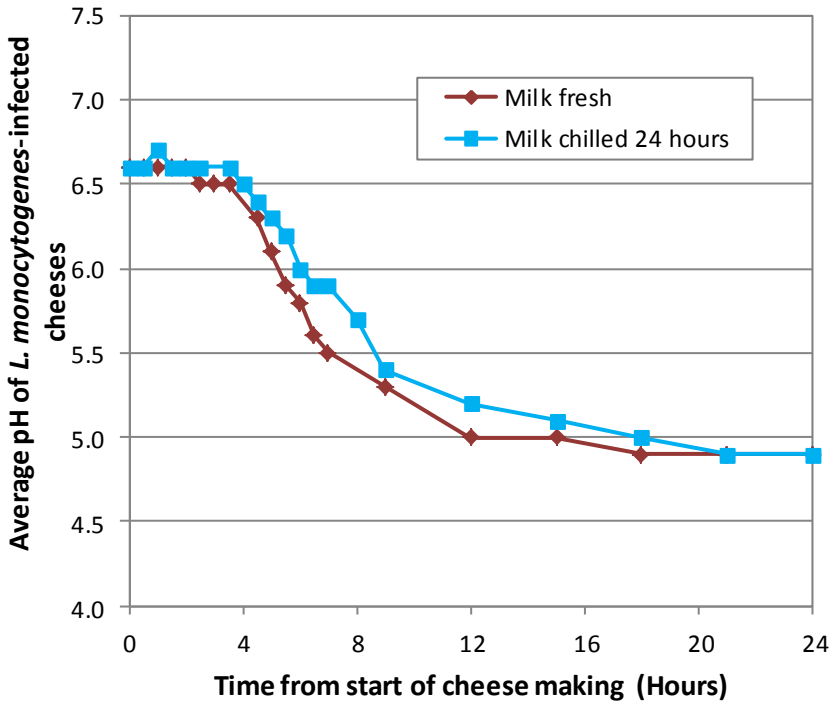
3.5.1 Microbiological analysis of raw milk and raw milk control cheeses

There were no *L. monocytogenes* detected in any of the raw milk samples arriving at ESR. Two other bacterial species, present in the raw milk and consistently present on the ALOA plates from the control cheeses (*Staphylococcus scuiri* and *Enterococcus faecalis*), were identified by 16S rRNA sequencing. These species were only rarely isolated from the *L. monocytogenes*-inoculated cheeses, and only at the lower dilutions. The bacterial colonies were clearly distinguishable from *L. monocytogenes.*

3.5.2 pH values of cheeses during laboratory manufacture

pH readings were taken as described in Table 1. The pH of raw milk arriving at ESR was 6.6. The pH decreased during the manufacturing period (up to 24 hours) on average from 6.59 (± 0.02) to 4.87 (± 0.06) in un-chilled milk (T0-T24: Day 1) and from 6.64 (± 0.01) to 4.91 (± 0.02) in chilled milk (T0-T24: Day 2) (Figure 3). As the pH ranges of each group of cheese (fresh: C1, C2, C3 chilled: C5, C6, C7 and control C4, C8) were similar at each time point, the mean values for the *L. monocytogenes*-infected cheeses only were used for generating points for the graph. The individual and mean pH results for all groups highlighted the consistency of the cheese making process and demonstrated that the introduction of *L. monocytogenes* did not change the pH profile during cheese manufacture. Chilled milk had a slightly higher pH during hooping (6-8 hours after the start of manufacture) however this was within the variation shown between individual cheeses using the same milk (data not shown). It was also almost identical to the pH profile obtained during the manufacturing stage of the cheese ripening trial using fresh milk (Experiment 2), (data not shown) demonstrating that a degree of variation can occur between cheeses made under slightly different conditions.

Figure 3. Mean pH values following laboratory manufacture of *L. monocytogenes*-infected cheeses made with raw un-chilled (fresh) or chilled milk.



NB: data for control cheeses not shown as pH values fell within the ranges defined above

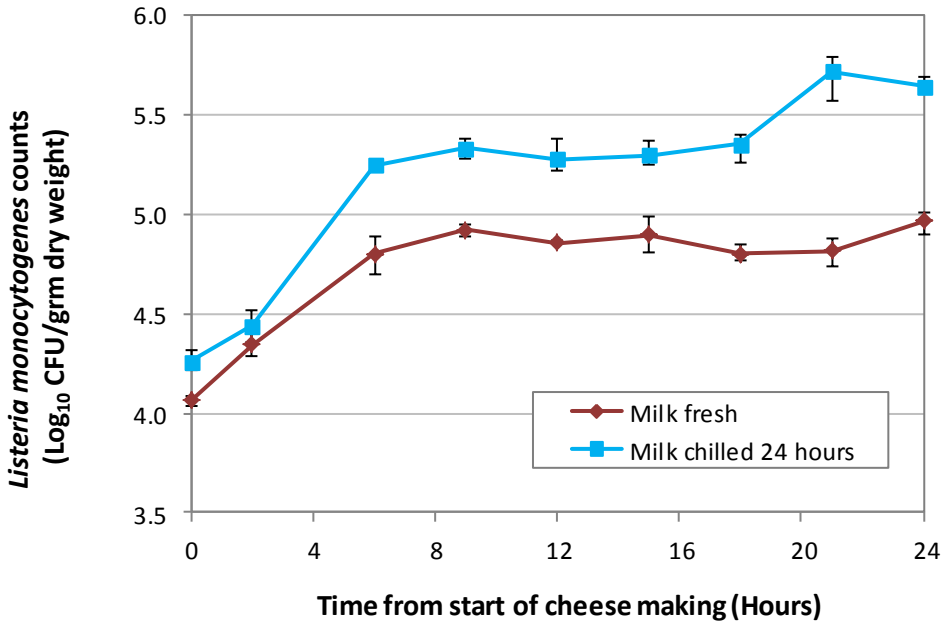
3.5.3 *L. monocytogenes* growth and survival during the laboratory manufacture of raw milk soft cheeses

A cocktail of *L. monocytogenes* isolates, at a final concentration of 3.13 $\log_{10}$ CFU/ml was added to warm, raw milk immediately after filtration in the laboratory. Samples were taken for microbiological analyses as described in Table 1. The data presented in Figure 4 represents the mean bacterial counts taken from triplicate samples (3 plates per sample) and the bars represent the range of values obtained by the three cheeses within each group (Un-chilled (fresh) milk or milk chilled for 24 hours). No *L. monocytogenes* were detected in either of the control cheeses at any time point.

There was some growth of *L. monocytogenes* during the 24 hours of chilling at 4°C resulting in 0.12 to 0.25 $\log_{10}$ CFU/g higher *L. monocytogenes* concentration at T0 for the milk samples inoculated and chilled prior to cheese making. There appeared to be slightly more growth of *L. monocytogenes* during the early stages of cheese manufacture (washing and early hooping)

using the chilled, compared with the un-chilled, milk. Taking into account the overnight growth of bacteria in the chilled milk, this represented an increase of approximately 0.25 log_{10} CFU/g. There was very little change in the survival rate of *L. monocytogenes*, for both milk types, between 6 and 18 hours from the start of cheese making with the higher concentration of *L. monocytogenes* (approx. 0.5log_{10} CFU/g difference) being detected in the cheese made from chilled milk. The bacterial concentration in the cheeses made from chilled milk increased between 18 hours and 21 hours after manufacture giving a difference of between 0.9 and 0.67 log_{10} CFU/g (when comparing cheeses made from chilled versus un-chilled milk) at T21 and T24 hours respectively.

Figure 4. Mean *L. monocytogenes* counts following laboratory manufacture of cheeses made with either raw, un-chilled (fresh) (C1, C2, C3) or chilled (C5, C6, C7) milk.



3.5.4 Bacterial entrapment within curds and whey portions of cheeses

At T3.5hrs, bacterial counts were quantified separately from the curds and whey portions of each cheese. The results suggest that most of the bacteria were concentrated in the curd portion and were therefore not discarded when the whey was removed. As such, bacteria in the curds have the potential to survive and multiply as the cheese manufacture and ripening stages progress (Table 3).

Table 3. *L. monocytogenes* concentration within the curds and whey portions of each cheese sampled at T3.5 hours after the start of cheese making.

	Cheese number							
	Un-chilled milk				Chilled milk			
	C1	C2	C3	C4	C5	C6	C7	C8
Curds (log_{10} CFU/g)	5.49	4.68	4.73	No sample	5.28	5.43	5.33	0
Mean curds (log_{10} CFU/g)	5.00				5.35			
Whey (log_{10} CFU/g)	4.58	4.42	4.17	No sample	4.76	4.69	4.56	0
Mean whey (log_{10} CFU/g)	4.39				4.67			

3.6 *L. monocytogenes* growth and survival during laboratory manufacture and ripening of raw milk soft Brie-like cheeses in the presence or absence of *P. roqueforti* (Experiment 2)

Raw milk for cheese manufacture and ripening was used un-chilled, at 32°C, immediately after filtration and inoculation. Cheeses were either made with or without the addition of the blue mould *P. roqueforti.* A flow chart detailing experimental conditions used for the manufacture and ripening of cheeses in Experiment 2 is presented in Appendix 1.

3.6.1 Cheese cave conditions for ripening and visualisation of ripening cheeses

A data logger was placed in the cheese cave prior to the start of ripening to ensure that the temperature and relative humidity levels were optimum for maturation (13°C/85% humidity) (Figure 5). Photographs of a ripening cheese (at Day 7) and visualisation of internal mould growth (at Day 28) are shown in Figure 6. Saline washes of cheeses, at the start of ripening, and the use of UV light in the cheese cave had the effect of reducing, but not preventing, surface mould growth over time.

Figure 5. Cheese ripening room: data logger entry showing temperature (°C) and relative humidity levels for the month of May 2014.

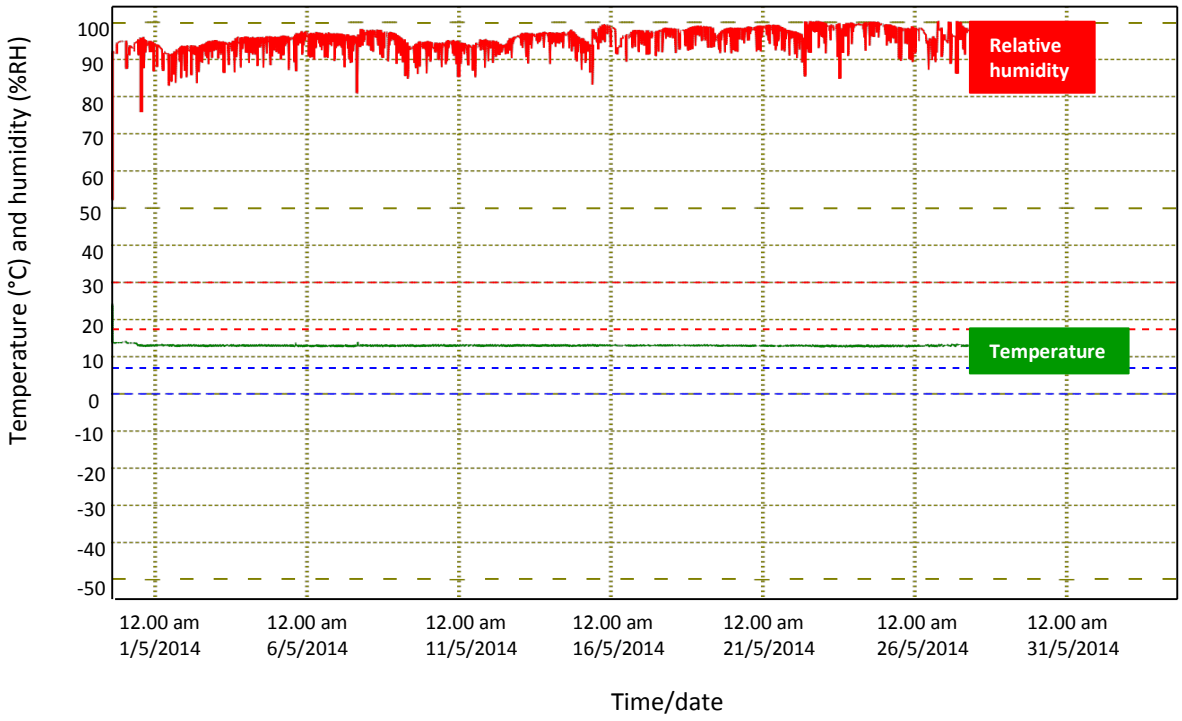


Figure 6. Day 7 cheese ripening (A), sampling portions with (B) and without (C) internal mould growth at Day 28 after manufacture.

3.6.2 pH values during laboratory manufacture and ripening of soft Brie-like cheeses

pH readings were taken as described in section 3.5.6.1. The pH of raw milk arriving at ESR was 6.6. During the manufacturing stage of cheese making, the pH of the three groups of cheeses:

L. monocytogenes plus *P. roqueforti* (C1, C2, C3); *L. monocytogenes* no *P. roqueforti* (C4, C5): no *L. monocytogenes* or *P. roqueforti* (C6) were within 0.1 of each other at every time point and as such, these results have not been presented. The pH profile of the cheeses for the ripening experiments, up to T24 hours after manufacture, was also almost identical to those obtained during the cheese making experiments described above (in Experiment 1), and presented in Figure 3.

There was little change in the pH values for all cheeses during the first two weeks of ripening with pH ranges between 4.8 and 5.5 on Day 7 and 4.9 and 5.6 on Day 14. From day 21 to day 42 of ripening, the pH of the cheeses varied considerably depending on whether 1) *P. roqueforti* was added during manufacture 2) sampling was from the inside or outside portions of the cheeses and 3) internal sampling was associated with areas of mould growth (C1, C2, C3 only). Cheeses with added *P. roqueforti* attained pH values in the range of 5.4 to 7.8 (inside) and 5.9 to 7.3 (outside) after six weeks, compared to 4.9 to 6.6 (inside) and 5 to 6.3 (outside) for cheeses with no added mould (excluding the control cheese). The pH measurements associated with the inside and outside of individual cheeses (C1-C5) are presented in Figure 7. For the cheeses with added *P. roqueforti* the pH plotted at time 21 to 42 days is for areas with visible mould. The pH values associated with the inside of cheeses 1 to 3 appeared to be marginally higher when taken from areas containing mould, compared to those areas that did not contain internal mould, although there was considerable variation between individual cheeses (Figure 8). After 21 days, all the visible mould pH samples had a pH value greater than 6.5.

Figure 7. pH values, from the inside and outside of individual cheeses, following laboratory manufacture and ripening of *L. monocytogenes*-infected cheeses made from raw fresh milk with or without the addition of *P. roqueforti*.

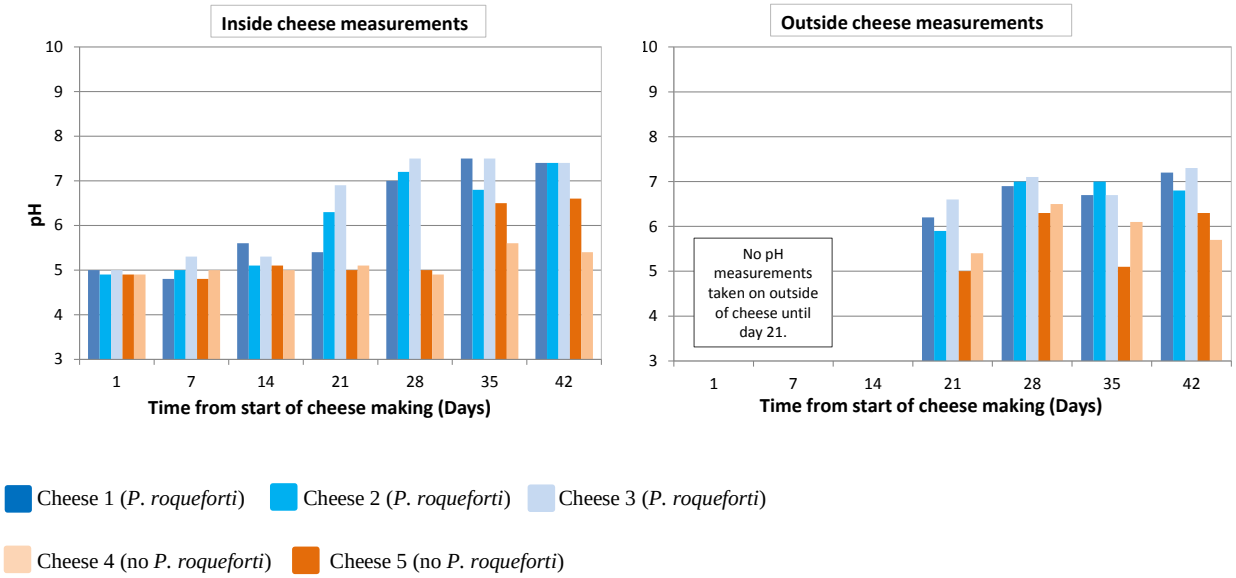
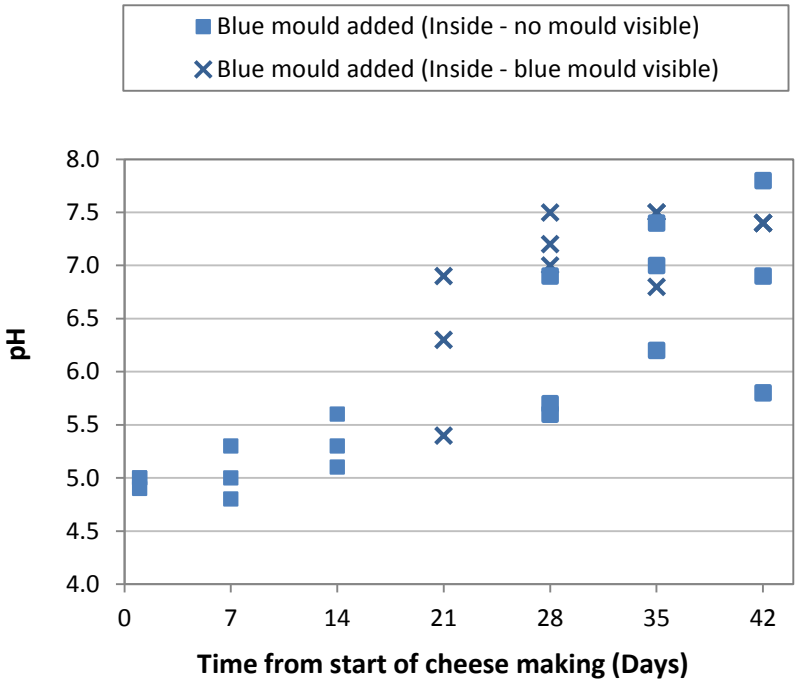


Figure 8. pH values, associated with or without blue mould, taken from the inside of cheeses (C1, C2, C3).

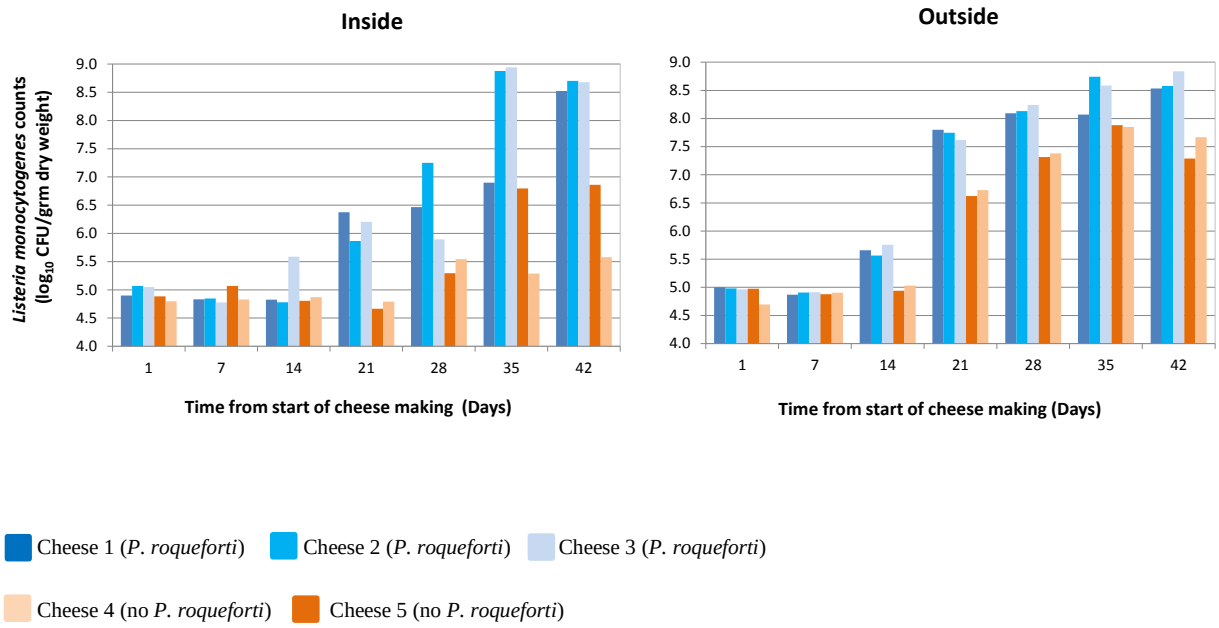


3.6.3 *L. monocytogenes* growth and survival during laboratory manufacture and ripening of soft cheeses

A cocktail of *L. monocytogenes* isolates, at a final concentration of 3.30 $\log_{10}$ CFU/ml was added to the warm, raw milk immediately after laboratory filtration. Samples were taken for microbiological analyses as described in Table 1 and section 3.5.6.2. The data presented in Figure 9 represents the mean *L. monocytogenes* counts taken from triplicate samples (3 plates per sample) either from the inside or the outside portions of the cheeses. No *L. monocytogenes* were isolated from the control cheese at any point during manufacture or ripening (data not shown).

During the cheese manufacturing stages (up to 24 hours) the concentration of *L. monocytogenes* was similar in all of the test cheeses (C1-C5) with an average increase in bacterial growth of 0.96 $\log_{10}$ CFU/g and 0.77 $\log_{10}$ CFU/g in the presence or absence of *P. roqueforti* respectively (data not shown). The growth rate of *L. monocytogenes* during this time period was similar to that quantified during the cheese manufacturing experiments using fresh milk (Experiment 1) (Figure 4). *L. monocytogenes* counts began to increase in the cheeses with added mould between days 7 and 14 in the outside cheese portions and between days 14 and 21 in the inside portions. By contrast, the growth of *L. monocytogenes* was slower in the cheeses with no added mould with bacterial counts increasing between days 14 and 21 in the outside portions and days 21 and 28 in the inside portions. By day 42 of ripening, there was a higher concentration of *L. monocytogenes* associated with the cheeses with added mould from all portions (8.52 $\log_{10}$ CFU/g to 8.84 $\log_{10}$ CFU/g) compared to cheeses with no added mould (5.58 $\log_{10}$ CFU/g to 7.67 $\log_{10}$ CFU/g). These differences were greater in the portions associated with the inside of the cheeses (Figure 9).

Figure 9. Quantification of the growth and survival of *L. monocytogenes* following laboratory manufacture and ripening cheeses made with raw fresh milk with or without the addition of *P. roqueforti*.



3.6.4 Water activity measurements during ripening of soft cheeses (Experiment 2)

Samples for water activity readings were taken at T0 hrs, T6 hrs, T24 hrs and thereafter weekly until 6 weeks post manufacture. As the cheeses aged, the water activity became lower. However, there were no differences in the readings associated with each of the cheese groups (Table 4).

Table 4. Water activity results associated with individual cheeses following manufacture and ripening.

	T0hrs	T6hrs	T24hrs		Day 7		Day 14		Day 21		Day 28		Day 35		Day 42	
dH_2O	1.00	1.00	1.00		1.00		1.00		1.00		1.00		1.00		1.00	
Sat. NaCl	0.75	0.75	0.75		0.75		0.75		0.75		0.75		0.75		0.75	
			in	out	in	out	in	out	in	out	in	out	in	out	in	out
C1	0.99	1.00	0.99	0.99	0.97	0.96	0.96	0.97	0.96	0.97	0.96	0.97	0.95	0.95	0.93	0.93
C2	1.00	1.00	0.99	0.99	0.96	0.96	0.97	0.97	0.97	0.97	0.96	0.96	0.94	0.95	0.92	0.93
C3	1.00	0.99	0.99	0.99	0.96	0.96	0.96	0.98	0.96	0.97	0.96	0.97	0.96	0.95	0.94	0.94
C4	1.00	1.00	0.99	1.00	0.96	0.96	0.96	0.96	0.97	0.97	0.97	0.97	0.95	0.95	0.94	0.94
C5	1.00	1.00	0.99	0.99	0.97	0.97	0.96	0.97	0.97	0.97	0.97	0.97	0.95	0.96	0.94	0.94
C6	1.00	0.99	0.99	0.99	0.96	0.96	0.98	0.98	0.97	0.97	0.97	0.97	0.94	0.94	0.95	0.94

'In' and 'out' refer to' inside' and 'outside' measurements from cheeses

dH_20 and Sat. NaCl refer to the negative and positive controls respectively used at each time point.

Shaded cells represent cheeses with added mould.

4 DISCUSSION

4.1 *L. monocytogenes* presence in cheese and raw milk

There are many reports of listeriosis outbreaks associated with the consumption of contaminated cheese. The majority are associated with high moisture cheeses and often those prepared in the absence of added starter culture during fermentation (Paulin *et al*., 2014). *Listeria* may get in raw milk directly from the infected animal or due to contamination during milking or milk storage. Factors which may result in *Listeria* being present in cheeses include poor hygienic practices (Leedom 2006), bacterial shedding from mastitic animals (Hunt *et al*., 2012), biofilm formation (Ho *et al*., 2007), pasteurisation failure, post-pasteurisation contamination, the introduction of contaminated ingredients, unsafe handling and storage conditions (Oliver *et al*. 2005., De Cesare *et al*., 2007., D'Amico and Donnelly 2008., King *et al*., 2014., Melo *et al*. 2015., Tiwari *et al*. 2015).

4.2 *L. monocytogenes* isolate selection and growth in raw and pasteurised milk

In the current study, initial screening of a range of *L. monocytogenes* isolates was undertaken to ensure that the cocktail for use in the cheese making experiments contained serotypes representing a variety of environmental sources, including dairy. Of the eight isolates obtained initially, five were deemed suitable candidates for further use based on the available clinical history, morphological and physical growth characteristics (Table 2). All of the *L. monocytogenes* serotypes represented in the final cocktail have previously been associated with outbreaks of listeriosis where cheese was implicated as a vehicle (Bille *et al*., 2006, Fretz *et al*., 2010, Johnsen *et al.*, 2010, Koch *et al*., 2010, De Castro *et al*., 2012).

All isolates selected demonstrated growth in raw and pasteurised milk which is consistent with that reported in the literature (Alavi *et al*., 1999; Pitt *et al*., 1999).

Competing microflora or other inhibitory factors, such as acetic or other organic acids produced by the background flora present in raw milk might limit the growth of *L. monocytogenes.* The inhibitory effect depends on differences in natural raw milk microflora and growth conditions used. Results could also be influenced by the particular isolate of *L. monocytogenes* used.

For example, Pitt et al (1999) demonstrated optimal anti-*Listeria monocytogenes* activity in raw bovine milk at 37ºC for up to 55 hours after inoculation Storage of raw milk at 4ºC for 4-6 days and incubation of milk at lower temperatures have been shown to decrease this activity. By contrast, the reduced number of competing organisms in pasteurised milk might offer a greater opportunity for pathogens to grow or survive (Gay and Agmar, 2005).

In the present growth experiments, there was no evidence that the raw milk inhibited the growth of *L. monocytogenes* comparing with growth observed in the pasteurised milk. This suggests the absence of anti-listerial activity against competing microflora in the raw milk used in this experiment.

4.3 The effect of chilling of inoculated milk on the subsequent growth of *L. monocytogenes*

The experiments in this study showed the behaviour of *L. monocytogenes* was unaffected by a period of chilling at 4°C for 24 hours after inoculation. Growth in milk held at 32°C for 8 hours immediately after inoculation, was similar to growth in the same milk that had been subjected to a period of chilling before the growth experiment.

The *L. monocytogenes* isolate cocktail was found to have similar counts in samples taken immediately after inoculation of the warm milk and after the milk had been chilled for 24 hours (3.31 and 3.35 $\log_{10}$ CFU/ml respectively). This result agrees with a recent review which concluded that at 4ºC no *L. monocytogenes* isolates exceeded 1 $\log_{10}$ growth in milk before 5 days (King *et al*., 2014). Brouillaud-Delattre *et al*., (1997) reported that no growth was observed in the first 24 hours when two day old raw milk was stored at 4°C, irrespective of whether the *L. monocytogenes* cells were in the exponential or stationary growth phases at innoculation. In another study, raw milk taken from an Italian vending machine, was inoculated with *L. monocytogenes* at a mean concentration 2.23 $\log_{10}$ CFU/ml (SD 0.03 CFU/ml). The mean concentration of *L. monocytogenes* after 27.5 hours was similar (2.35 $\log_{10}$ CFU/ml; SD 0.2 CFU/ml) to that at inoculation (Giocometti *et al* 2012).

A study using naturally-infected raw milk from a cow with listerial mastitis (concentration of approximately 4 $\log_{10}$ CFU/ml) investigated the growth of *L. monocytogenes* stored at 4°C

(Farber *et al.* 1990). No growth was observed after one day of storage. The lag time ranged from 3-5 days followed by an average generation time of 25.3 hours.

In our 32°C growth experiments, no (or minimal) growth was observed for some *L. monocytogenes* isolates after 2 (5/5 isolates using chilled milk and 4/5 isolates using un-chilled milk) or 4 hours. After 8 hours growth 1.5 to 2 log CFU/ml had been observed for all isolates in experiments using un-chilled milk and chilled prior to the growth experiment milk.

In a study by Brouillauid-Delattre *et al.* (1997) four isolates of *L. monocytogenes* incubated in raw milk at 30°C showed growth over a 6 hour period of 0.9 to 1.3 log CFU/ml. After the initial two hours the observed growth was between 0.1 and 0.6 log CFU/ml. These growth patterns are similar to the growth patterns seen in the current experiments.

These results suggest that *L. monocytogenes* can survive at 4°C in raw bovine milk, but growth is minimal. At cheese making temperatures of 30-32°C, modest growth of up to 2 log CFU/ml could be expected for some isolates when starting with chilled or un-chilled milk.

4.4 *L. monocytogenes* growth during cheese manufacture using un-chilled or chilled milk

In New Zealand, there are strict regulations in place that govern the time and temperature variables which may be applied to raw milk prior to further processing. In the current study, cheeses were manufactured using *L. monocytogenes*-inoculated raw milk that was either used un-chilled within 2 hours from milking or that had been chilled to 4°C for 24 hours prior to cheese manufacture. The pH range of the samples taken during the cheese making process fell within expected limits (pH 6.5- 4.6 during 24 hours) and is in agreement with another study (Pitt *et al*., 1999) confirming that the addition of *L. monocytogenes* does not alter the pH range of the cheese during manufacture.

A small increase in *L. monocytogenes* concentration was observed at the sampling point at the end of the raw milk chilling stage (between 0.12 and 0.25 $\log_{10}$ CFU/g). Subsequent pathogen growth and survival during the cheese making process was greater in cheeses made from chilled, rather than un-chilled milk (Figure 4). Further growth was observed at 21 hours in the cheese made from chilled milk. At 21 and 24 hours after the start of cheese manufacture, the

concentration of *L. monocytogenes* was greater by 0.9 and 0.67 CFU/g in the cheeses made with chilled milk, compared to un-chilled milk.

The growth observed in these experiments is in contrast to some reports in the literature. In a recent challenge trial, the effect of microbial interactions on the growth and survival of *L. monocytogenes* was investigated during the initial fermentation step of cheese manufacture using pre-conditioned (chilled) raw and pasteurised milk. The authors found no significant changes in the concentration of *L. monocytogenes* over a 12 hour period suggesting that if the pathogen is present in the milk prior to cheese making it will not grow during fermentation. Schvartzman *et al*. (2011) demonstrated no growth during the first five hours of manufacture of raw milk cheeses, but growth when using pasteurised milk. The authors suggested the increased concentration of background microflora associated with raw milk had the effect of limiting the growth of both starter bacteria and *L. monocytogenes.*

The results from the current study (Table 3) indicated the majority of bacteria prior to hooping were concentrated within the cheese curds, rather than being discarded in the whey. Similar observations have been demonstrated by Papageorgiou and Marth (1998) who found that *L. monocytogenes* were primarily entrapped within the curd portion before hooping. The whey contained only 3.6% of the cells in the initial inoculum. The concentration of *L. monocytogenes* in Table 3 from both the curds and whey portions were on average slightly higher in the cheeses made from chilled rather than un-chilled milk, although this was within the variation in the concentration of *L. monocytogenes* derived from the individual cheeses. The concentration in the curd ranged from 4.68 to 5.49 $\log_{10}$ CFU/g in cheeses made with un-chilled milk compared to 5.28 to 5.43 $\log_{10}$ CFU/g in cheeses made with chilled milk. This difference in entrapment of the bacteria in the curds is within the expected variation due to the experimental process.

The current study has shown that while examples discussed in the literature have suggested that *L. monocytogenes* is not likely to grow during the initial stages of raw milk cheese manufacture, it is possible for growth of 1 log CFU/g to occur. These differences in results suggest that there is a possibility of a delay in the growth of *L. monocytogenes* depending on the composition of the background flora in the raw milk.

In this study chilling the milk before heating to 32°C during manufacture did not induce a growth lag or reduce the growth relative to using un-chilled milk. The potential for growth and any lag in growth rate once subjected to initial cheese manufacturing temperatures also depends on other factors including individual strain or isolate of *L. monocytogenes* used and the unique properties of raw milk itself which may contribute to pathogen inhibition (Lake *et al*., 2005, Jordan *et al*., 2010, Schvartzman *et al*., 2010, King *et al*., 2014).

In the current experiments the growth rate difference of *L. monocytogenes* between chilled and un-chilled milk was greatest between the 2 and 6 hour sampling points. During this time, the pH values (Figure 3) were similar, but with the chilled milk cheeses having a slightly higher pH (6.03 chilled compared to 5.78 un-chilled at 6 hours). It is not known if this small pH difference would be sufficient to result in the difference in *L. monocytogenes* concentration (Figure 4). After 8 hours the pH was close to 5.5 and further dropped to 5. As 5.5 to 5 is in the region of the growth/no growth boundary for *L. monocytogenes* in cheeses during ripening (Paulin *et al*. (2014), pH may be the main driving force for stationary concentration after 8 hours.

At the 21 and 24 hour sampling times, the concentration of *L. monocytogenes* in the chilled milk cheeses had increased by approximately 0.5 $\log_{10}$ CFU/g relative to the preceding sampling points. This increase is consistent across the three cheeses using chilled milk, suggesting that the result is not due to a localised event in one cheese. It might be that bacteria which previously experienced temperature shock became more resistant to the harsh environmental conditions during cheese making.

Chilling the milk before manufacture did not reduce the growth relative to using un-chilled milk. While the data presented represents samples taken from a single milk batch only, the results are similar to those obtained in the growth curve experiments and demonstrate that chilling of milk prior to cheese manufacture under these conditions did not induce a lag effect either due to chilling or the change in temperature from 4°C to 32°C.

4.5 Effect of *P. roqueforti* on the growth and survival of *L. monocytogenes* during cheese ripening

Blue cheeses have a heterogeneous micro-environment with gradients in pH, salt and water activity, as well as localised differences in the vicinity of mould growth. During the ripening

process, the actively growing *P. roqueforti* catabolises the lactic acid to carbon dioxide, and excretes enzymes that contribute to the production of ammonia. Both of these mechanisms increase the pH of the cheese in the regions of mould growth during ripening (Cantor *et al*. 2004; Kinstedt, 2014).

The heterogeneous environment in terms of pH during ripening was clearly seen in the cheeses produced in the laboratory, as shown in Figure 8. For example, in cheese 3 at day 28 of ripening, an interior sample of cheese with no mould visible had a pH of 5.7 and in an area of interior cheese with mould visible the pH was 7.5. The heterogeneity in pH values throughout the experimental cheeses with added *P. roqueforti* (Figure 8) have influenced the growth rate of *L. monocytogenes* in different areas of the interior of the cheese and might explain the inter-cheese variation in *L. monocytogenes* concentration observed at 14 to 35 days of ripening.

A pH of less than 5.0 is likely to inhibit growth or decrease the concentration of *L. monocytogenes* (reviewed by Paulin *et al*., 2014; Rosshaug *et al*., 2012). Actively growing blue mould will increase the pH making the environment more amenable to the growth and survival of *L. monocytogenes* (Papageorgiou and Marth 1989, Paulin *et al*., 2014). In the experimental cheeses, the pH in the interior sample of the *P. roqueforti* cheeses increased from 5 at day 14 to between 5.3 and 6.9 at day 21 and remained above 6.7 at day 28 and after.

In the current study, the data suggests a relationship between the higher pH values associated with the addition of the blue mould *P. roqueforti* and the increased concentration of *L. monocytogenes*. While it is not possible to conduct strict statistical tests for this correlation given the experiment design, the trends in the pH and *L. monocytogenes* concentration time series suggest that *L. monocytogenes* survival and growth was mostly governed by the pH in the cheese (Figures 7 and 9).

In a similar study evaluating the fate of *L. monocytogenes* during the manufacture and ripening of pasteurised milk blue cheese, the authors concluded that when the pH dropped below 5 there was a decrease in the concentration of *L. monocytogenes* by an average of 2.68 $\log_{10}$ CFU/g (Strain *California*) and 2.65 $\log_{10}$ CFU/g (Strain *Scott A*) over the first 50 days of ripening, compared to bacterial populations in one-day-old cheeses. However, as the mould concentration and the pH increased, the inactivation of *L. monocytogenes Scott A* stopped during the rest of

the 140 day ripening period. *L. monocytogenes California* after a stable concentration from 50 to 80 days continued to decrease in concentration. (Papageorgiou and Marth 1989). The pH of the *Scott A* cheeses (pH 6 to 6.5) where observed to be higher than the *California* cheeses (pH 5.25-5.75) between 60 and 140 days of ripening which may explain the difference in growth behaviour. The results of Papageorgiou and Marth (1989) are different to the results obtained from the current study which demonstrated an increase in the concentration of *L. monocytogenes* throughout the ripening process once the pH began to rise and the pH of the blue cheeses reached 7. The addition of a high concentration of salt during the manufacturing stages in the Papageorgiou and Marth experiment, could result in a slower rate of blue mould growth during ripening, which would influence changes in pH and therefore pathogen survival.

Another study examining the fate of *L. monocytogenes* during the manufacture and ripening of Camembert cheese concluded that *L. monocytogenes* was able to grow after 18 days of ripening and that bacterial growth paralleled the increase in pH of the cheese during the ripening period (Ryser and Marth, 1987). In agreement with the findings of the current study, these authors also observed that *L. monocytognenes* counts increased most rapidly in surface samples whereas growth was markedly slower in interior cheese samples which had a lower pH value.

pH may not be the only mechanism influencing growth of *L. monocytogenes* during ripening. *L. monocytogenes* may be inhibited by fatty acids produced as the *P. roqueforti* mould starts to grow. *P. roqueforti* secretes extracellular lipases which break down components of fat globules to produce fatty acids (Cantor *et al*., 2004). Over time the fatty acids are further broken down by the *P. roqueforti* into methyl ketones or to non-volatile calcium salts as the pH rises. The transient inhibition may partially explain the lower concentration of *L. monocytogenes* in the interior of the cheese in the fourth week of ripening compared to the fifth week.

High concentrations of the fatty acids dodecanoic acid and tetradecanoic acid have been found in the mould areas of ripened blue-veined Brie cheeses (Kindelerer et al 1996). Dodecanoic acid dissolved in butter oil at pH 7.0 at a temperature of 9.6°C was found to reduce the growth in butter oil of a strain of *L. monocytogenes* which had been previously been found in a blue mould cheese. After 4 days, the increase in *L. monocytogenes* concentration in the presence of dodecanoic acid was less than 3 $\log_{10}$ CFU/ml compared to a growth of 5 $\log_{10}$ CFU/ml in controls with no added acid. Decanoic and tetradecanoic acid also reduced the growth of

L. monocytogenes, but to a lesser extent, with a growth of approximately 4 $\log_{10}$ CFU/ml. The growth of ten strains of *L. monocytogenes* in the presence of hexanoic and octanoic acids at pH of 5 to 5.5 has also been tested at 20°C (Kinderlerer and Lund 1992). Inhibition of growth was observed at acid concentrations comparable to those found in blue mould cheeses.

Geisen *et al* (1988) conducted inhibition experiments of *P. roqueforti* on *L. monocytogenes* strains at 37°C for 2 days. While this temperature was high compared to that used for the cheese ripening experiments, this work showed that the inhibition of growth was dependent on the strain of both organisms.

In commercial mould-ripened cheese production, brining is usually for a longer duration and at a higher salt concentration than that used in the current ripening experiments. Under commercial conditions, salting or brining creates a NaCl gradient from the surface of the cheese to the core which equilibrates slowly during ripening. Growth of *P. roqueforti* is inhibited on the cheese surface due to the high salt concentration but as the salt diffuses into the centre of the cheese, and the salt concentration is lower, the blue mould is able to grow. Hence the mould grows from the interior of the cheese to the exterior (Cantor *et al*. 2004). In the current study, *P. roqueforti* was also observed to grow from the inside of the cheese to the exterior. There was a degree of surface mould present on the experimentally-ripened cheeses in the current study. The salt rinses and brine concentration were not sufficiently concentrated to inhibit mould growth.

Another possible hurdle to *L. monocytogenes* growth is water activity due to the cheese drying out during ripening. Throughout the manufacturing and ripening process, the water activity of the cheeses with added *P. roqueforti* was the same as cheeses with no added mould (Table 4). The water activity of cheeses during ripening was 0.96-0.97 between day 7 and day 28 of ripening, dropping to 0.92 to 0.95 by day 42. A water activity of 0.92 is documented as the no-growth boundary for *L. monocytogenes* for pH above 4.2 (Ross, 2011). Hence, the reduced water activity in isolation would not have totally prevented the growth *L. monocytogenes* during ripening. The addition of *P. roqueforti* did not change the water activity of the cheese.

5 CONCLUSION

This study was conducted to determine whether currently-permitted cooling and storage conditions of raw milk, for raw milk cheese manufacture, have an effect on the growth and survival of *L. monocytogenes* during manufacture of soft Brie-like cheese. Secondly, this work aimed to evaluate how the growth of *L. monocytogenes* during ripening was influenced by the addition of *P. roqueforti* which is commonly used for internal mould ripening.

Contrary to suggestions in the literature (Schvartzman *et* al., 2011; Withers and Couper, 2012) that *L. monocytogenes* growth would not occur during the early stages of cheese manufacture with raw milk, growth of 0.73-1 $\log_{10}$ CFU/g was observed in the first 6 hours of cheese manufacture using both milk that was un-chilled and milk that had been chilled for 24 hours at 4°C.

This experiment has shown that the growth of *L. monocytogenes* in cheeses made with chilled milk can be equal or higher than that seen in cheeses made with milk used shortly after milking and not chilled. This suggests that chilling raw milk prior to cheese making cannot be considered as a measure to reduce pathogen growth.

The presence of *P. roqueforti* in the soft cheese resulted in changes to the growth patterns of *L. monocytogenes* compared to cheese with no added mould.

In the *P. roqueforti* cheeses, an increase in *L. monocytogenes* concentration was observed 7 days earlier than in cheeses with no *P. roqueforti*. Growth was observed between day 14 and 21 inside the *P. roqueforti* cheeses, compared to 21 to 28 days inside cheeses with no *P. roqueforti*. On the outside of the cheeses, growth was observed between 7 and 14 days for cheeses with added blue mould and 14 and 21 days for cheeses with no mould added. These population increases corresponded to increases in the pH of the cheese. The pH of the cheese increased due to actively growing mould catabolising lactic acid to carbon dioxide, as well as excreting enzymes that produce ammonia.

An increased maximum sustained concentration of *L. monocytogenes* during ripening was also observed inside cheeses with *P. roqueforti* (8.5 to 8.9 $\log_{10}$ CFU/g) compared to cheeses with no added *P. roqueforti* (5.5 to 6.5 $\log_{10}$ CFU/g).

The difference in growth behaviour of *L. monocytogenes* in the two cheese types is mostly due to the changes in pH. However, the growth of *L. monocytogenes* inside the *P. roqueforti* cheeses may also be inhibited by fatty acids produced as the *P. roqueforti* mould starts to grow. The fatty acids are themselves broken down during cheese ripening, so only provide a transient inhibition of *L. monocytogenes*.

6 REFERENCES

Alavi, H. H., V. M Pure, S. J. Knabel, R. H. Mohtar and R. C. Whiting (1999). Development and validation of a dynamic growth model for *Listeria monocytogenes* in fluid milk. Journal of Food Protection **62** (2) 170-176.

Bille, J., D. S. Blanc, H. Schmid, K. Boubaker, A. Baumgartner, H. H. Siegrist, M. L. Tritten, R. Lienhard, D. Berner, R. Anderau, M. Treboux, J. M. Ducommun, R. Maliverni, D. Genne, P. Errard and U. Waespi (2006). Outbreak of human listeriosis associated wtih Tomme cheese in Northwest Switzerland, 2005. Eurosurveillance **11**(6): pii 633.

Bovill, R., J. Bew, N. Cook, M. D. Agostino, N. Wilkinson, J. Baranyi. (2000). Predictions of growth for *Listeria monocytogenes* and *Salmonella* during fluctuating growth. International Journal of Food Microbiology **59**: 157-165.

Brouillaud-Delattre, A., M. Marie, C. Collette, C. Mattei and C. Lahellec (1997). Predictive microbiology of dairy products: Influence of biological factors affecting growth of *Listeria monocytogenes*. Journal of the Association of Official Analytical Chemists **80**(4): 913-919.

Cantor, M. D., T. van den Tempel., T. Fox and Y. Ardo (2004). Blue Cheese, p 175-189. *In* Fox, P. F., McSweeny, P. L. H., Cogan, T. M. Guinee, T. P (*Ed*), Cheese Chemistry, Phyics and Microbiology. 3rd Edition, volume 2. Major cheese groups . Elsevier Academic Press, SanDiego, CA.

D'Amico, D. J. and C. W. Donnelly (2008). Enhanced detection of *Listeria* spp. in farmstead cheese processing environments through dual primary enrichment, PCR, and molecular subtyping. Journal of Food Protection **71**(11): 2239-2248.

De Castro, V., J. M. Escudero, J. L. Rodriguez, N. Muniozguren, J. Uribarri, D. Saez and J. Vazquez (2012). Listeriosis outbreak caused by Latin-style fresh cheese, Bizkaia, Spain." Eurosurveillance **17**(42): pii 20298.

De Cesare, A., G. Manfreda, M. Macrì and C. Cantoni (2007). Application of automated ribotyping to support the evaluation of *Listeria monocytogenes* sources in a Taleggio cheese producing plant. Journal of Food Protection **70**(5): 1116-1121.

Farber, J. M., G. W. Sanders and J. I. Speirs (1990). Growth of *Listeria monocytogenes* in naturally-contaminated raw milk. Lebensmittel-Wissenschaft & Technologie **23**(3): 252-254.

Fretz, R., J. Pichler, U. Sagel, P. Much, W. Ruppitsch, A. T. Pietzka, A. Stöger, S. Huhulescu, S. Heuberger, G. Appl, D. Werber, K. Stark, R. Prager, A. Flieger, R. Karpíšková, G. Pfaff and F. Allerberger (2010). Update: Multinational listeriosis outbreak due to 'Quargel', a sour milk curd cheese, caused by two different *L. monocytogenes* serotype 1/2a strains, 2009-2010. Eurosurveillance **15**(16): pii 19543.

Gay, M. and A. Amgar (2005). Factors moderating *Listeria monocytogenes* growth in raw milk and in soft cheese made from raw milk. Lait **85**(3): 153-170.

Gaya, P., C. Saralegui, M. Medina and M. Nunez (1996). Occurrence of *Listeria monocytogenes* and other *Listeria* spp. in raw caprine milk. Journal of Dairy Sci **79**(11): 1936-1941.

Giacometti, F., A. Serraino, G. Finazzi, P. Daminelli, M. N. Losio, M. Tamba, A. Garigliani, R. Mattioli, R. Riu and R. G. Zanoni (2012). Field handling conditions of raw milk sold in vending machines: experimental evaluation of the behaviour of *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella* Typhimurium and *Campylobacter jejuni*. Italian Journal of Animal Science **11**(2): 132-136.

Groves, R.D. and Welshimer, H.D. (1977). Separation of pathogenic from apathogenic *Listeria monocytogenes* by three *in vitro* reactions. Journal of Clinincal Microbiology **5**: 559-563.

Harvey, J. and A. Gilmour (1992). Occurrence of *Listeria* species in raw milk and dairy products produced in Northern Ireland. Journal of Applied Bacteriology **72**(2): 119-125.

Ho, A. J., R. Ivanek, Y. T. Grohn, K. K. Nightingale and M. Wiedmann (2007). *Listeria monocytogenes* fecal shedding in dairy cattle shows high levels of day-to-day variation and includes outbreaks and sporadic cases of shedding of specific L. monocytogenes subtypes. Preventive Veterinary Medicine **80**(4): 287-305.

Hunt, K., N. Drummond, M. Murphy, F. Butler, J. Buckley and K. Jordan (2012). A case of bovine raw milk contamination with *Listeria monocytogenes*. Irish Veterinary Journal **65**(1): 13.

IDF (1982). International Dairy Federation Standard 4A: Cheese and processed cheese. Determination of the total solids content (reference method). Brussels, Belgium.

IDF (1987). International Dairy Federation standard 21B: Milk, cream and evaporated milk. Determination of total solids content (reference method). Brussels, Belgium.

Johnsen, B. O., E. Lingaas, D. Torfoss, E. H. Strom and I. Nordoy (2010). A large outbreak of *Listeria monocytogenes* infection with short incubation period in a tertiary care hospital. Journal of Infection **61**(6): 465-470.

Jordan, K., M. S. Schvartzman, A. Maffre, M. Sanaa, F. Butler, U. Gonzales-Baron and F. Tenenhaus-Aziza (2010). Predictive models developed in cheese for growth of *Listeria monocytogenes*. Australian Journal of Dairy Technology **65**(3): 150-152.

Karahadian, C. L., R.C (1987). Integrated roles of lactate, ammonia and calcium in texture development of mold surfaced ripened cheese. Journal of Dairy Science **70**: 909-918.

King, N., R. Lake and P. Cressey (2014). Risk profile: *Listeria monocytogenes* in raw milk. Client Report FW13052. A report for the New Zealand Ministry for Primary Industries., Institute of Environmental Science and Research.

Kinstedt, P. S. (2014). The basics of cheesemaking, p 17-38. *In* Donnelly, C. W. (*Ed*), Cheese Chemistry and microbes. ASM Press, Washington, DC.

Koch, J., R. Dworak, R. Prager, B. Becker, S. Briockmann, A. Wicke, H. SWichmann-Schauer, H. Hof, D. Werber and K. Stark (2010). Large listeriosis outbreak linked to cheese made from pasteurized milk, Germany, 2006-2007. Foodborne Pathogens and Disease **7**(12): 1581-1584.

Kowalik, J., A. Lobacz, A. S. Tarczynska and s. Ziajka. (2012). Graphic validation of growth models for *Listeria monocytogenes* in milk during storage. Milchwissenschaft. **67** (1): 38-42.

Lake, R., J. A. Hudson, P. Cressey and S. Gilbert (2005). Risk Profile: *Listeria monocytogenes* in low moisture cheeses. Client report FW0440. Christchurch, Institute of Environmental Science and Research (ESR) Limited.

Leedom, J. M. (2006). Milk of non-human origin and infectious diseases in humans. Clinical Infectious Diseases **43**(5): 610-615.

Lianou, A. and K. P. Koutsoumanis (2013). Strain variability of the behavior of foodborne bacterial pathogens: A review. International Journal of Food Microbiology **167**(3): 310-321.

Melo, J., P. W. Andrew and M. L. Faleiro (2015). *Listeria monocytogenes* in cheese and the dairy environment remains a food safety challenge: The role of stress responses. Food Research International **67**: 75-90.

Nightingale, K. K., Y. H. Schukken, C. R. Nightingale, E. D. Fortes, A. J. Ho, Z. Her, Y. T. Grohn, P. L. McDonough and M. Wiedmann (2004). Ecology and transmission of *Listeria monocytogenes* infecting ruminants and in the farm environment. Applied Environmental Microbiology **70**(8): 4458-4467.

Oliver, S. P., B. M. Jayarao and R. A. Almeida (2005). Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. Foodborne Pathogens and Disease **2**(2): 115-129.

Papageorgiou, D. K. and E. H. Marth (1989). Fate of *Listeria monocytogenes* during the manufacture and ripening of blue cheese. Journal of Food Protection **52**(7): 459-465.

Paulin, S. M. (2013). Growth of *Staphylococcus aureus* and toxin production during laboratory manufacture of a model, soft cheese. Client Report MFS/11/10. A report for the New Zealand Ministry for Primary Industries., Institute of Environmental Science and Research Limited.

Paulin, S. M. King, N. Lake, R. Cressey, P (2014). Risk profile: *Listeria monocytogenes* in cheese. Client Report FW13049. A report for the New Zealand Ministry for Primary Industries., Institute of Environmental Science and Research Limited.

Pitt, W. M., T. J. Harden and R. R. Hull (1999). Antibacterial activity of raw milk against *Listeria monocytogenes*. Australian Journal of Dairy Technology **54**: 90-93.

Ross, T. (2011). Challenge testing of microbiological safety of raw milk cheeses: the challenge trial toolkit. Prepared for Ministry for Primary Industries as a MAF Technical Paper: No 2011/51 by Tom Ross, University of Tasmania. ISBN: 978-0-478-38437-9 (online).

Rosshaug, P. S., A. Detmer, H. Ingmer and M. H. Larsen (2012). Modeling the growth of *Listeria monocytogenes* in soft blue-white cheese. Applied and Environmental Microbiology **78**(24): 8508-8514.

Ryser, E. T.and E. H. Marth (1987). Fate of *Listeria monocytogenes* during the manufacture and ripening of Camembert cheese. Journal of Food Protection **50**(5) 372-378.

Schvartzman, M. S., X. Belessi, F. Butler, P. Skandamis and K. Jordan (2010). Comparison of growth limits of *Listeria monocytogenes* in milk, broth and cheese. Journal of Applied Microbiology **109**: 1790-1799.

Schvartzman, M. S., A. Maffre, F. Tenenhaus-Aziza, M. Sanaa, F. Butler and K. Jordan (2011a). Modelling the fate of *Listeria monocytogenes* during manufacture and ripening of smeared cheese made with pasteurised or raw milk. International Journal of Food Microbiology **145, Supplement 1**(0): S31-S38.

Tiwari, U., E. Cummins, A. Valero, D. Walsh, M. Dalmasso, K. Jordan and G. Duffy (2015). farm to fork quantitative risk assessment of *Listeria monocytogenes* contamination in raw and pasteurised milk cheese in Ireland. Risk Analysis **35**(6) 1140-1153.

Wiedmann, M. and K. G. Evans (2011). Diseases of Dairy Animals: Infectious Diseases - Listeriosis. Encyclopedia of Dairy Sciences (Second Edition). J. W. Fuquay. San Diego, Academic Press**:** 184-189.

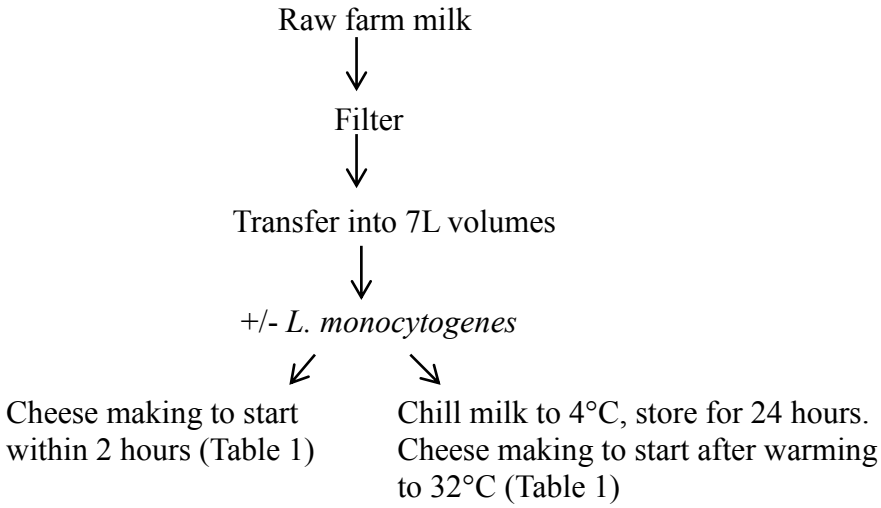
Willman, N. and Willman, C. (2005). Home cheesemaking. Australia, Neil and Carole Willman.

Withers, H. and Couper, J. (2012). Effect of microbial interactions on pathogen growth and survival during fermentation of raw milk. AgResearch, Prepared for Ministry for Primary Industries.

Xanthiakos, K., D. Simos, A. S. Angelidis, G. J. Nychas, K. Koutsoumanis. (2006). Dynamic modeling of *Listeria monocytogenes* growth in pasteurised milk. Journal of Applied Microbiology **100** (6): 1289-12988.

Appendix 1: Flow chart detailing experimental conditions used for the manufacture and ripening of raw milk, soft cheeses.

Experiment 1 (Cheese manufacture)



Experiment 2 (Cheese manufacture and ripening)