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Manatū Ahu Matua

Quantitative Assessment of Microbiological Safety of Raw Milk Cheeses Manufacturing

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Prepared for the Ministry for Primary Industries
by Dr Chawalit Kocharunchitt and Assoc Prof Tom Ross

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Quantitative Assessment of Microbiological Safety of Raw Milk Cheeses Manufacturing

The project "Quantitative Assessment of Microbiological Safety of Raw Milk Cheeses Manufacturing" has been undertaken by the University of Tasmania to assess the relative safety of analogous cheeses made from raw or pasteurised milk, and to evaluate the use of predictive models to support food safety risk management decisions about specific cheeses and cheese making processes.

A challenge study is one of the most reliable approaches to validation the cheese manufacturing process. As part of this project, a series of challenge studies involving production of Wensleydale, Cheddar, Gouda, Feta (matured at two temperatures) and double-cream Brie cheeses from both raw and pasteurised milk deliberately contaminated with mixtures of *Listeria* spp. and *E. coli* strains were conducted.

The main conclusion from the studies was that the safety of cheeses is primarily determined by the hygienic quality of the milk used to make the cheese, not by the ability of the process to eliminate pathogens, with the possible exception of Feta-style cheeses and cheeses that involve a cooking step (e.g., hard grating cheese). While good hygiene during manufacture of cheeses made from pasteurised milk can ensure the safety of final product, the risk of pathogen contamination in soft cheeses made from raw milk cannot be mitigated by the process.

Key observations include:

- There were no systematic differences in the ecology or fate of either *Listeria* spp. and *E. coli* in cheeses made from raw or pasteurised milk. While the growth of cheese pathogens in raw milk compared to pasteurised milk during acidification appeared to be slightly slower, pathogen die-off during ripening in raw milk cheeses was slightly faster. In total, the process results in a similar net change in pathogen concentration irrespective of the state of the milk.
- Rate of acidification can affect the potential for growth of pathogens during acidification and curd formation. Faster acidification results in less growth during cheese making.
- Pathogen growth and acidification are slower in cheese made using goats milk compared to cows' milk when manufactured using similar starter cultures and incubation temperatures.
- Pathogen inactivation rates in cheeses at normal maturation and storage temperatures are very slow requiring weeks to months under commercial conditions to reduce pathogen loads by one order of magnitude, irrespective of the milk species or state.
- The effect of temperature is such that an increase of 5°C in maturation temperature approximately doubles the inactivation rate, again irrespective of the milk species or state.

University of Tasmania

Tasmanian Institute of Agriculture - School of Land and Food

Quantitative Assessment of Microbiological Safety of Raw Milk Cheese Manufacturing.

(Final Report, Project 11877, 11 November 2014)

prepared for

New Zealand Ministry for Primary Industries

by

Dr. Chawalit Kocharunchitt and Assoc. Prof. Tom Ross

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University of Tasmania

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Project Summary

Project Overview and Approaches

This project, jointly-funded by NZMPI with Victorian Department of Health, Australia aimed to:

i) assess the relative safety of analogous cheeses made from raw or pasteurised milk
ii) increase understanding of the ecology and fate of microbial pathogens in cheeses
iii) evaluate whether existing predictive models can provide sufficiently reliable predictions to support food safety risk management decisions about specific cheeses and cheese making processes based on raw or pasteurised milk.

While other pathogens may be present in milk or cheese, the project focussed on the ecology and fate of *Listeria monocytogenes* and *Escherichia coli* as the pathogens of greatest interest and relevance in cheeses from the perspective of public health.

The project involved characterisation of physico-chemical properties of nearly 100 commercial cheeses, studies of pathogen survival in simulated cheese systems and, finally, challenge studies involving two strains of each challenge organism, in five varieties of cheese, with batches made from both raw milk, or the same milk after pasteurisation.

The study undertaken represents one of the largest and most detailed studies of pathogen survival in raw milk cheeses, and has generated rigorous scientific data enabling direct comparison of survival of *L. monocytogenes* and *E. coli* in equivalent raw or pasteurised milk cheeses. The data generated also allow changes in pathogen populations to be correlated with the changing physico-chemical properties of the cheese during processing and maturation.

Observations and Results

The results obtained suggest that there is no systematic difference in the ecology or fate of either of the two pathogens in cheeses whether made from raw or pasteurised milk but using otherwise identical methods and processes. While raw milk cheeses appeared to inhibit pathogen growth during acidification of the milk very slightly compared to the growth observed in pasteurised milk during those processes, the rate of pathogen die-off during ripening was slightly slower in raw milk cheeses. Consequently, these slight differences effectively 'cancel each other out' so that there are no differences in pathogen survival in the same cheese if made from raw or pasteurised milk.

The studies showed that rate of acidification can affect the potential for growth of pathogens during acidification and curd formation and that the observed growth was usually greater for strains of *E. coli* than for species of *Listeria*. There was also some evidence that milk type may affect the rate of growth: much slower pathogen growth, and slower acidification, was observed in a cheese made using goats milk with similar starter cultures and incubation temperatures as in some cows' cheeses made in the project.

The inactivation kinetics for *Listeria* spp. differed from those for *E. coli*. While both displayed biphasic inactivation kinetics, *E. coli* responses were characterised by a rapid initial inactivation phase followed by a slower phase. Conversely, *Listeria* spp. displayed a slow initial inactivation (a 'lag') but, subsequently, the rate of inactivation increased. Temperature was shown to have a consistent effect on inactivation rate and its *relative* effect was similar for all challenge organisms in all challenge media. Studies in milk-based broths with low water activity (a_w = 0.947), high and low pH, and high and low concentrations of undissociated lactic acid, suggest that the latter two factors can have a significant effect on inactivation rates but that, in most commercial cheeses, the levels are not sufficiently extreme for the effects to be noticeable. Brined cheeses, however, have lower pH and much higher undissociated lactic acid levels and faster pathogen inactivation rates were observed in Feta-style cheese than in other main categories of cheese.

Inactivation rates of pathogens in cheeses at normal maturation and storage temperatures are, however, very slow, requiring weeks to months under commercial conditions to reduce pathogen loads by one order of magnitude. At 15°C, the lag times for *Listeria* spp. were >2000 hours (>80 days). At 15°C, times for a one $\log_{10}$ reduction in *E. coli* in the second, slower, phase were typically 500-1000 hours (20-40 days). Thus, with the possible exception of Feta-style cheeses and cheeses that involve a cooking step (e.g., hard grating cheese), *the safety of cheeses is dominated by the hygienic quality of the milk used to make the cheese, not by the ability of the process to eliminate pathogens.* This was found to be equally true for cheeses made from pasteurised milk or raw milk. Given the growth/concentration of pathogens that occurred during tempering and curd formation, maturation times of 20–100 days are required to achieve "no net change" in pathogen levels (*i.e.*, compared to levels initially present in the milk), irrespective of cheese style (with the possible exception of brined cheeses). The effect of temperature is such that an increase of 5°C in maturation temperature approximately doubles the inactivation rate for either *E. coli* or the two *Listeria* spp. studied.

From the challenge trial data, 260 observations were used to assess performance of a number of mathematical models intended to be able to predict the potential for growth of *E. coli* or *L. monocytogenes* in foods. When growth was predicted to be possible, the *rate* of growth was predicted from other models. Use of a simple model based on the absolute limits of growth for each organism showed that the Hurdle Effect was evident in providing microbiological stability of cheeses, i.e., the combinations of temperature, salt, pH and organic acid concentrations inhibited growth more than any factor acting alone. Using more complex growth/no growth models that implicitly include the consequences of the 'Hurdle Effect' produced more 'correct' predictions of 'growth' or 'no growth' than the simple models. The models also predicted that changes in the pH of Brie cheeses during maturation would enable growth of *L. monocytogenes* to resume during ripening as was actually observed in the challenge trials. Most importantly, there were no predictions of 'no growth' if growth was, in fact, observed, i.e., the models were "fail safe".

From the survey of commercial cheeses, it was observed that there is variation in physico-chemical properties of cheeses of the same nominal style, but produced by different manufacturers. Equally, however, that there was variation between the same product from individual manufacturers made on different dates. Accordingly, any regulatory decisions based on the nominal physico-chemical properties of cheeses should also include allowance for the variability likely to occur.

Acknowledgements

The authors wish to sincerely thank the New Zealand Ministry of Primary Industry for the opportunity to undertake this project and their patience during its delayed completion.

We wish to acknowledge Ms. Kathleen Shaw for initiating the project and for her management and contributions to the initial parts of the project and also her successor, as project manager, Dr. Tanya Soboleva for her insights and intellectual contributions, ensuring that the project produced relevant results to advise effective food safety management.

Dr. Heather Haines is thanked for her support, advice and co-management of the joint project on behalf of Department of Health, Victoria.

Mr. Ashley McCoy, of Wicked Cheeses, Cambridge, Tasmania, provided expert advice and support for the preparation of the various cheeses constituting the challenge trials. His commercial perspectives were invaluable in the conduct of this study. Similarly, we thank Ms. Sharee McCammon, of the University of Tasmania's Central Science Laboratory, for her contributions as an experienced 'hobby' cheese maker, to making the cheeses for the challenge studies that were an integral part of this project.

We also thank Ms. Bianca Porteus, a PhD student and Ms. Caitlin Maney, an MSc student, at the School of Land and Food, University of Tasmania. They both toiled for long, and 'unusual', hours to assist in the making and analyses of cheeses in the challenge studies. Dr. Lyndal Mellefont provided expert technical advice for the design, conduct and analyses of microbiological aspects of challenge studies.

Finally, we wish to thank Dr. Joanna Jones, and Mr. Will Bignell and his extended family, both of whom provided raw bovine milk for these studies. We thank also Hans Stutz and Esther Haeusermann of Tongola Goat Products of Cygnet, Tasmania, for supplying raw goat's milk to us, even when it was in short supply for their own operations.

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CONTENTS

1 Physico-chemical characteristics of cheeses

A range of commercially available cheeses was purchased at various retail outlets within Hobart, Tasmania, Australia, over a period of approximately 14 months. New Zealand cheeses were sought, but were not commercially available and were not able to be supplied directly. The overall objective was to characterise the physico-chemical properties of cheeses that might affect the ecology of pathogenic bacteria that could be present in those cheeses. It was hypothesised that the fate of pathogens in cheese (e.g., growth, stasis, inactivation) might be related to the style of cheese, or to one or other specific characteristics, which could then be used to evaluate the safety of different styles/types of cheese without recourse to challenge tests. Accordingly, the variability among cheeses of similar types, as well as among the same cheeses from a single manufacturer over time, were also assessed to determine whether a particular designated style/name of cheese is associated with consistent physico-chemical parameters.

1.1 Physico-chemical properties of cheeses

Ninety-eight cheeses were characterized according to their pH, lactic and acetic acid content, water content and water activity. Methods of analysis are described in Appendix 1. The properties selected for measurement were those considered most likely to affect the fate of the target bacterial pathogens in cheese.

Cheeses purchased were grouped into six main categories:

i) Brined
ii) Cheddar ('hard')
iii) Hard-grating
iv) Internal-mould-ripened
v) Semi-hard
vi) Soft, surface-ripened

and another group of cheeses, simply designated as 'other', for those that did not readily fit into the categories listed above.

The classification of cheeses employed in this report is similar to that proposed by Ottogalli (2000) but with the "hard and extra hard" category considered in this study as two distinct categories (*i.e.,* 'Cheddar' and 'Hard Grating') due to the prevalence of Cheddar-style cheeses in the Australian and New Zealand markets. The categorisation is somewhat *ad hoc,* and subjective, due to the diversity of cheese styles and nomenclature. That diversity relates equally to the style nominated by the manufacturer: as shown below, there was diversity in the characteristics of cheeses with the same name but produced by different manufacturers.

Full results of analyses are presented in Appendix 2. Summary data, according to nominal cheese style, are presented in Table 1.1, overleaf. Figures 1.1 to 1.5 show the ranges of the measured parameters more explicitly according to nominal cheese style.

Table 1.1. Summary characteristics (mean, with standard deviation shown in italics) of 98 cheese samples grouped according to nominal style.

Cheese Style	pH	Water activity	Water content (% w/w)	Lactic acid (% w/w)	Acetic acid (% w/w)	Number of samples
Brined	4.41 *0.18*	0.96 *0.01*	53.86 *3.69*	0.90 *0.72*	0.06 *0.05*	12
Cheddar ("hard")	5.30 *0.20*	0.94 *0.02*	35.59 *3.19*	0.83 *0.41*	0.09 *0.08*	16
Hard-grating	5.38 *0.20*	0.90 *0.01*	36.21 *1.89*	1.02 *0.37*	0.10 *0.05*	6
Internal mould-ripened	6.24 *0.92*	0.93 *0.02*	44.94 *3.05*	0.52 *0.51*	0.10 *0.09*	12
Semi-hard	5.56 *0.22*	0.96 *0.01*	41.50 *3.35*	0.82 *0.56*	0.11 *0.12*	23
Soft, surface-ripened	6.46 *0.54*	0.98 *0.01*	48.83 *4.91*	0.07 *0.08*	0.04 *0.02*	24
Others	5.36 *0.19*	0.96 *0.02*	42.47 *11.73*	0.47 *0.17*	0.13 *0.10*	5

Figures 1.1 and 1.2, overleaf, present the range of pH and lactic acid in cheeses according to nominal style. From those figures it can be seen that brined cheeses have much lower pH than most other cheeses analysed and relatively high lactic acid concentrations. The pH observed is expected for this style of cheese (Fox *et al*., 2000). Conversely, soft, surface-ripened cheeses have higher pH and relatively lower lactic acid content, on average, than the other types of cheeses assessed. Internal-mould-ripened cheeses also tended to have higher pH. Mould- and surface-ripened cheeses, including those ripened by bacteria such as *Brevibacterium linens*, are expected to have higher pH due to proteolysis and catabolism of amino acids in the cheese caused by the introduced moulds/bacteria. (Proteolysis involves release of amine groups, which are 'basic' in chemical composition and cause the pH of the cheese to rise as proteolysis continues).

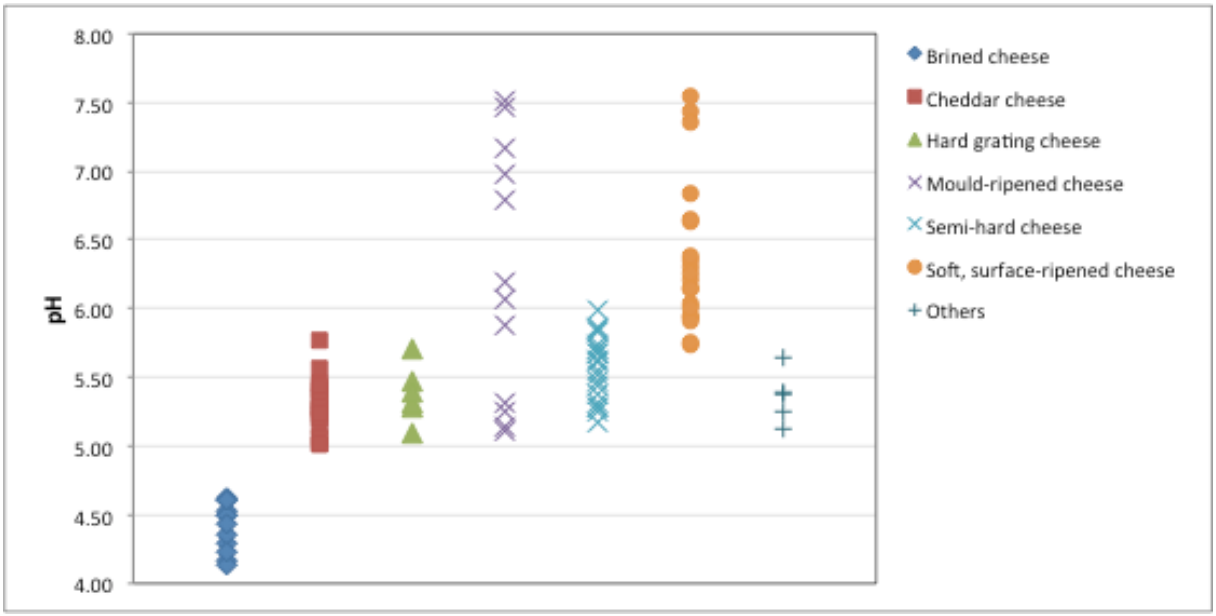


Figure 1.1. pH ranges of cheeses according to nominal style.

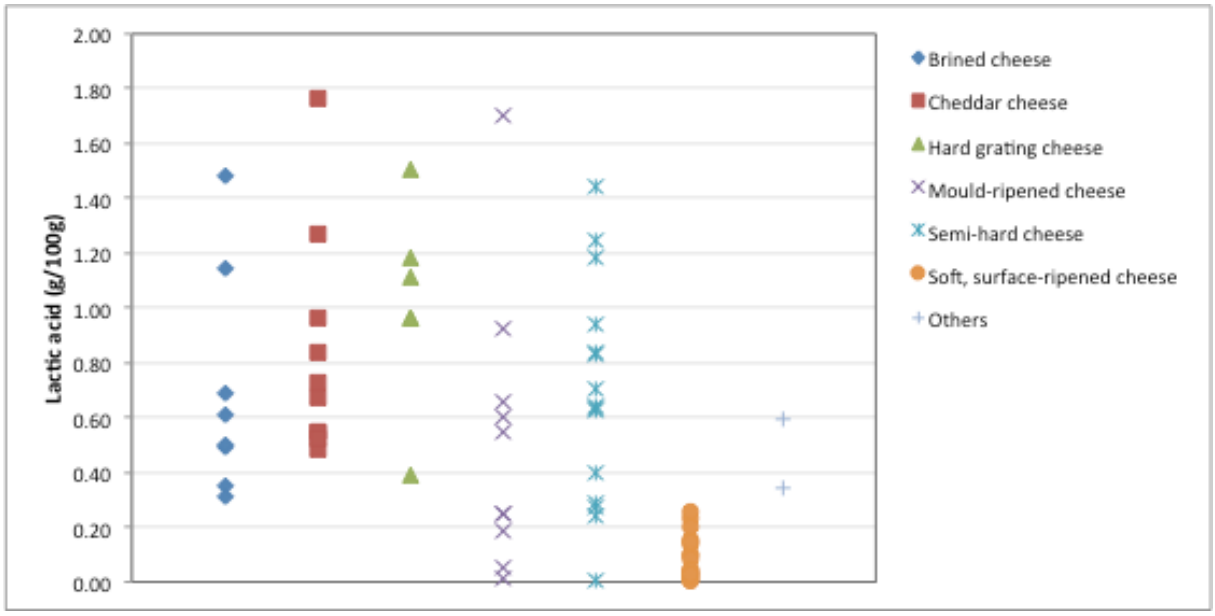


Figure 1.2. Lactic acid concentration ranges of cheeses according to nominal style.

As discussed later (*see* Section 2), in broths designed to emulate the physico-chemical conditions in cheeses, both *E. coli* and *Listeria* spp. (*L. monocytogenes* and *L. innocua)* appeared to have much faster inactivation rates in broths intended to emulate brined cheeses than all other cheese styles studied. The most unique aspect of brined cheeses (among the properties assessed) is that they have much lower pH than all other cheese styles considered. In previous studies on the effect of pH on inactivation of *E. coli* in fermented meats and other environments that precluded growth due to pH and/or water activity constraints (McQuestin *et al.*, 2009), inactivation rates were found not to

be correlated with pH. Those studies included inactivation rates assessed over a pH range of 2.8 to 6.1, but lactic acid concentration data were not included in that analysis (because lactic acid levels were not reported in most of the publications from which the inactivation data for that study were obtained). In other studies, McQuestin (2006) found no consistent effect of lactic acid (0 or 150 mM total concentration) on inactivation rates of *E. coli* at pH in the range 4.7 to 6.2. Organic acids inhibit microbial growth and it is observed that the undissociated form (*i.e.*, un-ionised) of the organic acid is a much better predictor of inhibition. This is generally attributed to the greater solubility of the undissociated form of the acid in lipids and, in consequence, its ability to enter the cell by passing through the lipophilic cell membrane. It is possible that undissociated lactic acid has an important role in determining inactivation rates and, accordingly, undissociated lactic acid concentrations were estimated from the pH and total lactic acid concentration data using the Henderson-Hasselbalch equation. The results are shown in Table 1.2.

Table 1.2. Undissociated lactic acid concentration (mean and standard deviations) of 98 cheese samples sorted according to nominal style.

Cheese Style	pH	Lactic acid (% w/w)	Undissociated Lactic acid (mM ± SD)	Number of samples
Brined cheese	4.41	0.90	20.5 ± 14.3	12
Cheddar cheese	5.30	0.83	3.6 ± 2.7	16
Hard grating cheese	5.38	1.02	3.8 ± 2.0	6
Mould-ripened cheese	6.24	0.52	1.92 ± 3.1	12
Semi-hard cheese	5.56	0.82	2.1 ±1.9	23
Soft, surface-ripened cheese	6.46	0.07	0.05 ± 0,08	24
Others	5.36	0.47	1.5 ± 1.6	5

It is apparent that the concentration of undissociated lactic acid in brined cheeses is nearly an order of magnitude higher than in other cheeses examined. Undissociated organic acid levels of 5 – 15 mM are typically sufficient, in the absence of other inhibitors to growth, to prevent microbial growth (Ostling and Linden, 1993; Presser *et al*., 1998; Lund and Eklund, 2000; Tienungoon *et al.,* 2000).

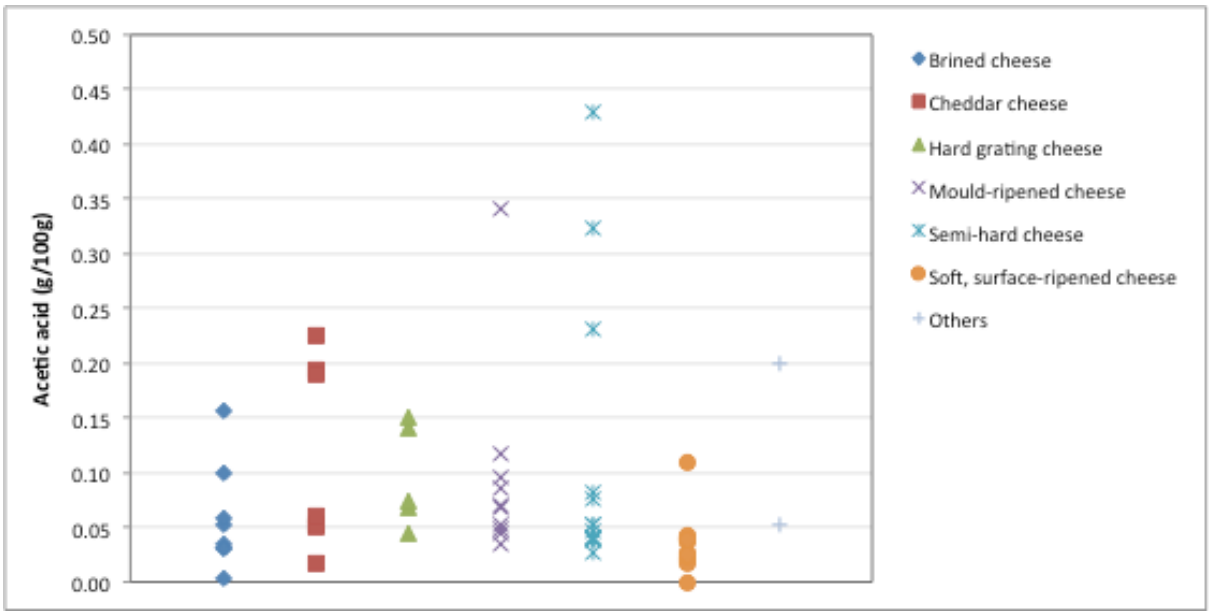


Figure 1.3. Acetic acid concentration ranges of cheeses according to nominal style.

As shown in Figure 1.3, total acetic acid concentrations are much lower than lactic acid concentration in cheeses. On a weight/weight basis, at a given pH, acetic acid is more inhibitory to microbial growth than lactic acid (Ostling and Lindgren, 1993; Young and Foegeding, 1993; Ouattara *et al*., 1997). Levels of undissociated acetic acid of the order of a few, or less, mM are usually sufficient to cause growth rate inhibition, and levels in the range up to 10 mM sufficient to *prevent* microbial growth (Ostling and Lindgren, 1993; Presser *et al.*, 1998; Ross *et al*., 2000; Mejlholm and Dalgaard, 2007). The analogous analyses to estimate undissociated acetic acid concentrations based on observed acetic acid concentrations and pH data (Figure 1.3) suggest undissociated acetic acid levels in the range 0.01 to 4 mM.

As evidenced in Figure 1.4 (overleaf), water activity levels are strongly related to the style of cheese, with harder cheeses generally having lower water activities. Water activity is affected both by the salt and water content of the cheese. Water contents of the cheeses are shown in Figure 1.5 (overleaf) and show similar trends, *i.e.,* lower water content generally correlates with lower water activity.

There is a variation in water activity (and water content) even among cheeses of similar style. Thus, while there is apparently a correlation between water activity and water content of the cheeses, the correlation is not strong statistically when evaluated on a cheese-by-cheese basis ($R^2 < 0.3$, *data not shown*). Water activity (a_w) < ~0.95 is sufficient to prevent growth of *E. coli* (Presser *et al.*, 1998*)* while a_w < ~0.925 is required to prevent growth of *L. monocytogenes* (Ross *et al.*, 2000) when all other factors are optimal for growth. If other factors contribute to inhibition of growth, less extreme water activity limits for growth may be observed.

Water content was relatively least variable among hard grating cheeses (Figure 1.5) though fewer samples were analysed. Among the parameters measured, organic acid levels (lactic acid, acetic acid) appeared the most variable when assessed as the variation around the mean value of the measured property for the specific cheese types.

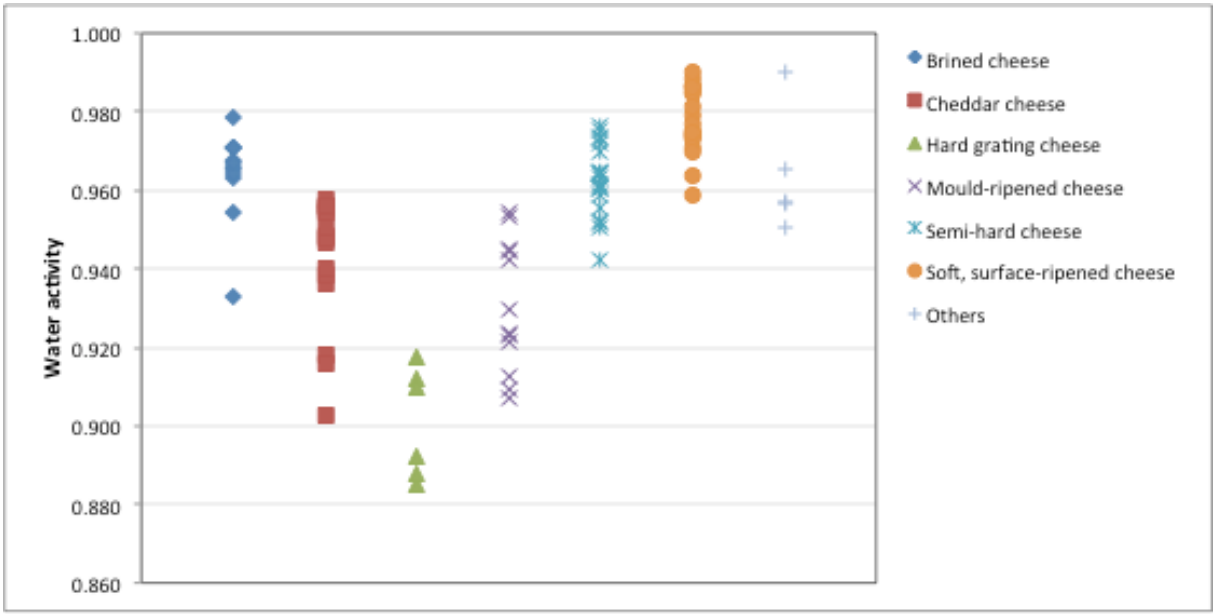


Figure 1.4. Water activity ranges of cheeses according to nominal style.

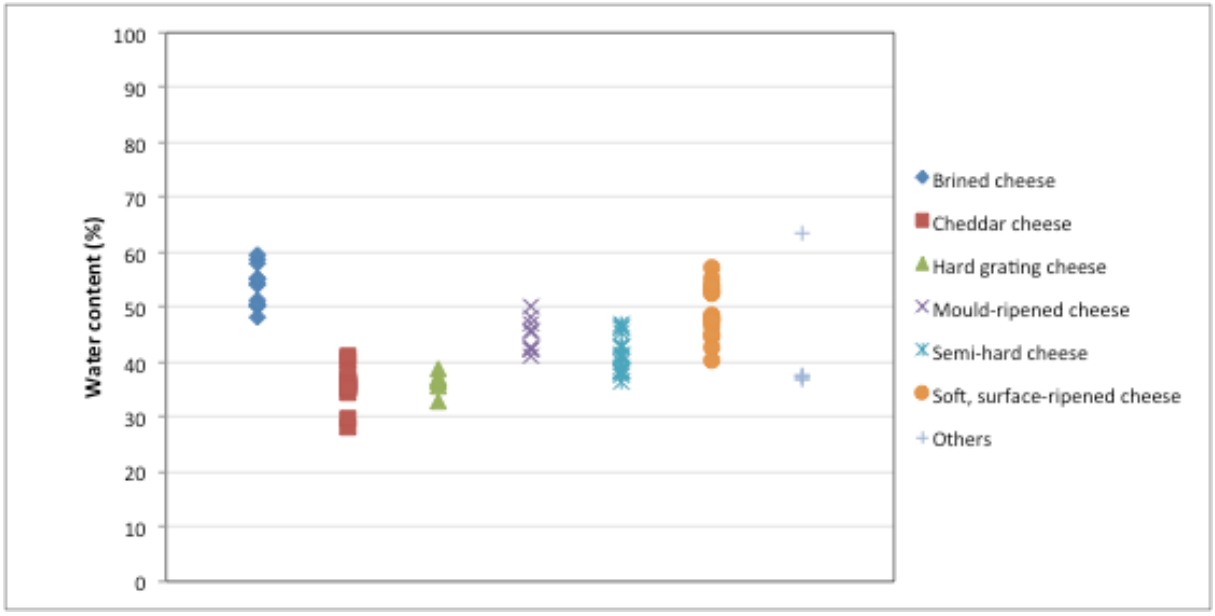


Figure 1.5. Water content ranges of cheese according to nominal styles

1.2 Variability between cheeses of the same style

The extent of variability (as assessed by the standard deviation of the data sets) of parameters within specific styles of cheese was relatively constant between groups, with the possible exception of the pH and lactic acid concentration of mould- and surface-ripened cheeses. Harder styles of cheeses tended to have less variable pH compared to mould- or surface-ripened cheeses. In harder cheese varieties, the pH tends to stabilize at an acidic level whereas, for reason outlined earlier, the pH of surface- and internal-mould-ripened cheeses tends to be more dynamic over the life of the cheese, initially declining during fermentation but rising again over time as proteolysis proceeds during maturation.

To assess the extent to which (nominal) cheese style relates to consistent and distinct physico-chemical characteristics of cheeses (meaning cheese style could be used as the basis of predictions of inactivation rates or limits to growth of pathogens in cheese), correlations between various properties of cheeses were examined. Figures 1.6 and 1.7 show correlations between pH and water activity of cheeses, and water activity and lactic acid concentrations of cheeses, respectively.

It is apparent (Figure 1.6) that water activity/pH combinations of different cheeses cluster according to cheese type, but for water activity/lactic acid concentration combinations this is less true. In other words, water activity/lactic acid concentration combinations are less characteristic for particular styles of cheese than are water activity/pH combinations. While other correlations were considered, the two combinations shown in Figures 1.6 and 1.7 are representative of the diversity of these characteristics within particular cheese styles and reinforce the observations of variability alluded to earlier. It should be noted, however, that there is a relatively strong correlation between pH and lactic acid content (Figure 1.8), as would be expected in a fermented product because the acidity of cheese is primarily derived by fermentation of sugars in milk (predominantly lactose) to low molecular weight organic acids (primarily lactic, acetic and, in some cheeses, propionic acids).

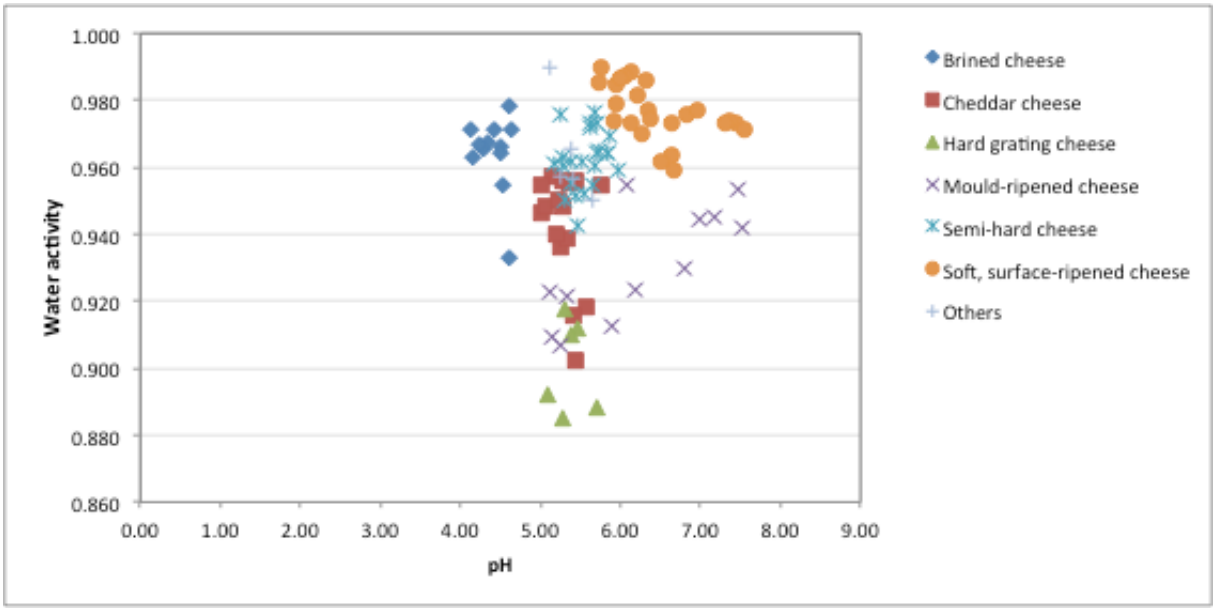


Figure 1.6. Correlations plot showing the relationship between water activity and pH for various styles of cheese.

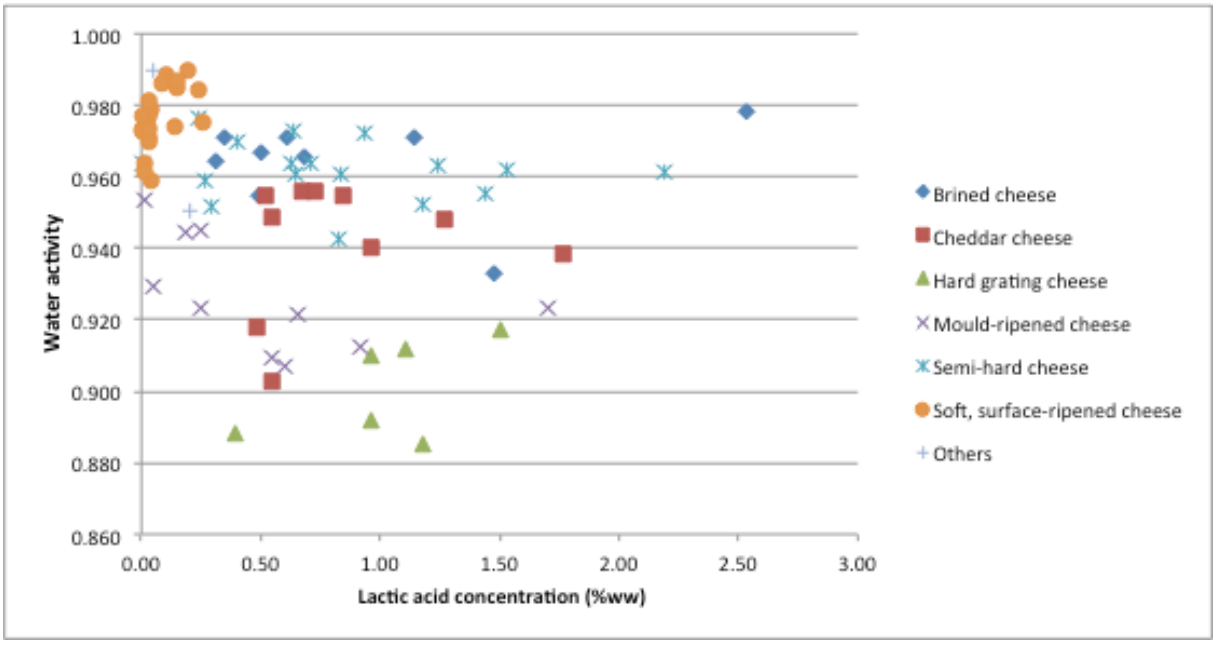


Figure 1.7. Correlations plot showing the relationship between lactic acid concentration and water activity for various styles of cheese.

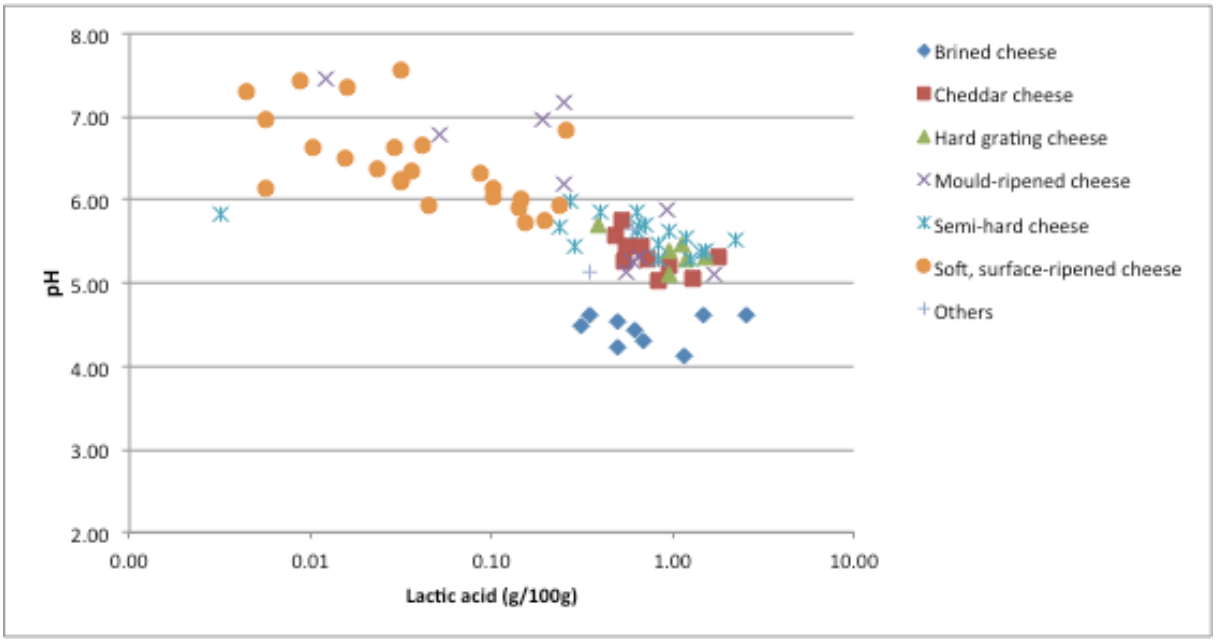


Figure 1.8. Correlations plot showing the relationship between lactic acid concentration and pH for various styles of cheese. The correlation co-efficient (R^2) between pH and log(lactic acid concentration) is 0.54.

1.3 Variability among batches of the same cheeses

To assess batch variability of a single cheese by a single cheese producer, several sets of samples of the same cheese were purchased at different times ensuring that different batches were sampled (e.g., as adjudged by batch code or 'best before' date). The replicated samples included:

- three temporally distinct replicates of an imported Danish blue cheese
- three temporally distinct replicates of an Australian-produced Gouda
- five temporally distinct replicates of an Australian-produced camembert
- three temporally distinct replicates of one brand of an Australian-produced "double brie"
- three temporally distinct replicates of another brand of an Australian-produced "double brie"
- three temporally distinct replicates of an Australian-produced brie

This combination of samples allowed the consistency of product parameters *among* cheese producers to be assessed, as well as the consistency of individual manufacturers. Representative results, shown in Figures 1.9 to 1.11, illustrate that the characteristics of cheeses from an individual producer are variable, but less variable than those of the cheese type as a whole.

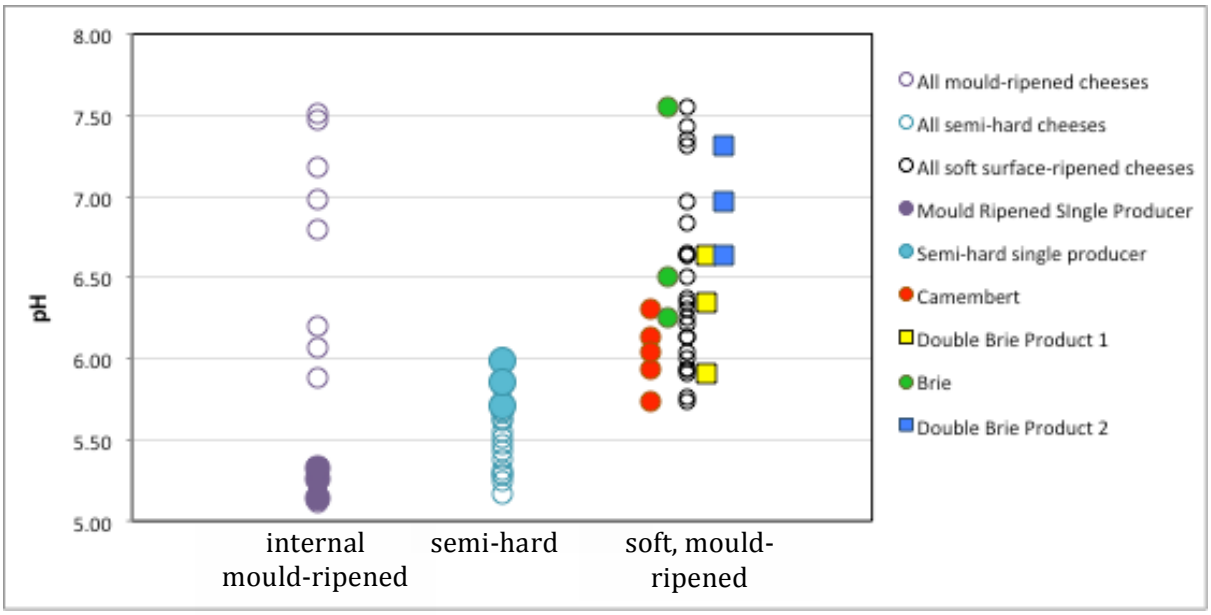


Figure 1.9. **Variability in pH of cheese of similar style from different producers and between batches from the same producer. Filled plot symbols represent analyses of cheese from different lots but from the same producer. Open symbols are analyses of cheese of the same style but from all producers assessed.**

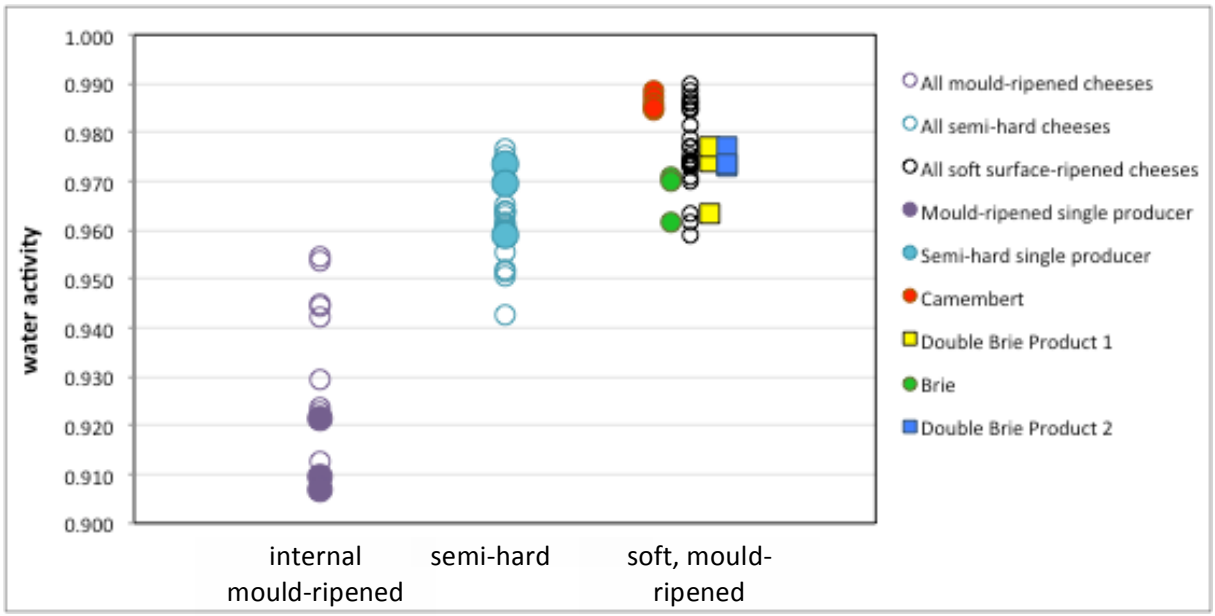


Figure 1.10. **Variability in water activity in cheeses of similar style from different producers and between batches from the same producer**. Filled plot symbols are analyses of cheese from different lots but the same producer. Open symbols (sometimes obscured by the analogous closed symbols) represent analyses of cheese of the same style but from all producers assessed.

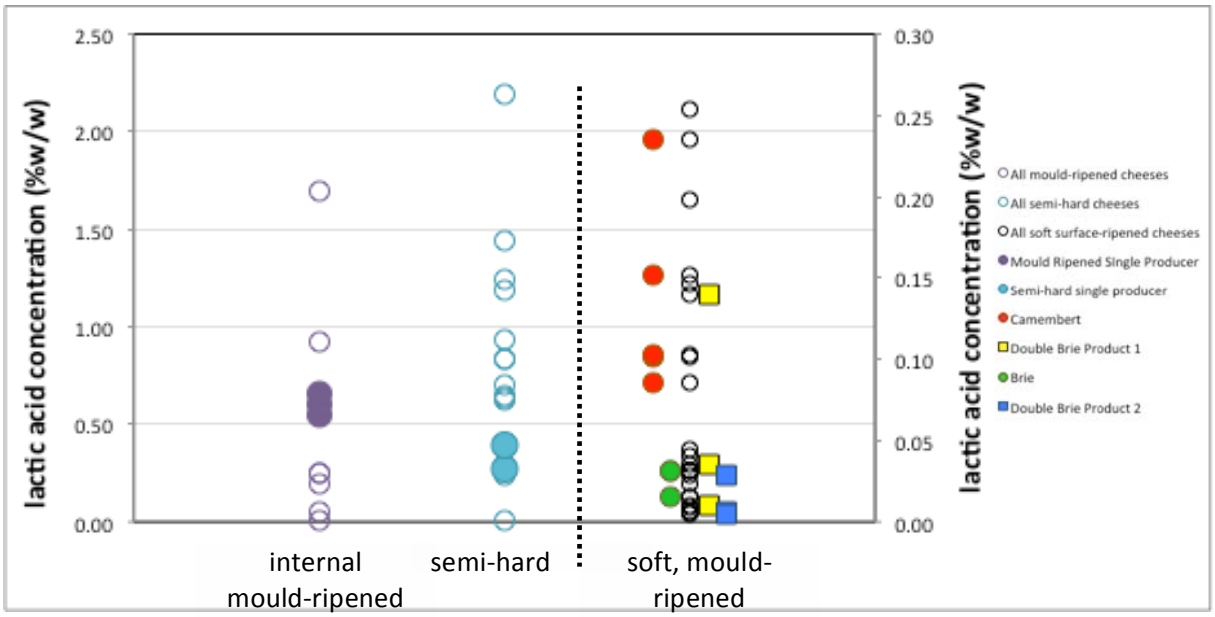


Figure 1.11. Variability in lactic acid concentration in cheese of similar style from different producers and between batches from the same producer. Filled plot symbols are analyses of cheese from different lots but the same producer. Open symbols are analyses of cheese of the same style but from various producers. The data for soft cheeses are plotted aginst the right-hand axis because the lactic acidc concentrations in soft, surface-ripened cheeses are much lower than for other cheese types assessed (*see also* Figure 1.2).

1.4 Conclusions

A large database of physico-chemical characteristics of cheeses, including replication, has been developed. The database enables resolution of questions about the reproducibility of characteristics among cheeses of similar styles and names.

While broad similarities are evident,from the data and analyses presented it is clear that there is a high degree of variability in physico-chemical parameters among cheeses of the same nominal style, and even those of the same 'name'. As such, the objective of being able to characterise the physico-chemical characteristics (and, by inference, the microbial ecology) of cheeses according to their name or nominal style does not seem to be feasible. Significant variation was found between batches of single types of cheese from single manufacturers. For example, the water activity of a Cheddar cheese from a single manufacturer ranged from ~0.958 to 0.972, a span of 0.014 a_w. Given that the a_w range for growth of *E. coli* is from ~0.95 to 0.999, this a_w variability represents 30% of the *E. coli* growth range and a potential three-fold difference in growth rate between the lower and upper limits of water activity range observed.

As will be demonstrated in subsequent Sections, pathogen inactivation rates were apparently much faster in broths intended to emulate brined cheeses and, from the data generated in characterising the cheeses, it was apparent that brined cheeses have much lower pH and much higher undissociated lactic acid concentrations than all other cheese types considered. Based on this observation, studies were initiated to determine whether low pH or very high undissociated lactic

acid concentration do cause significantly faster inactivation of bacterial pathogens than in other, common, fermented foods that have less extreme pH and undissociated lactic acid levels.

2 Inactivation kinetics of *Listeria* spp. and *E. coli* in broths intended to emulate cheeses

2.1 Objectives

Preliminary studies (data not shown) suggested that challenge trials with pathogen in cheeses would generate faster inactivation rate estimates than trials in simple broths that *emulate* cheeses. Accordingly, a broth-based model that would enable more data to be generated less expensively, would be expected to lead to a more robust model, than one generated by data obtained from challenge studies. Data generated from broth systems would provide 'fail-safe' or 'worst-case' predictions of the extent of pathogen inactivation over time. A broth model system, therefore, could be used to develop a *conservative* model to evaluate the microbiological safety of cheeses.

We conducted experiments to generate inactivation rate data for *Listeria* and *E. coli* in a range of "milk-based media" that emulated different cheese styles. The results are presented in this Section and also in Appendix 3.

2.2 Approach

A series of broths based on "milk-based medium" (1% full cream milk powder, 0.5% peptone and 0.3% yeast extract) were prepared with pH, water activity (a_w) and lactic acid levels representative of different types of cheese, based on the data presented in Table 1.1. The composition of these broths, given in Table 2.1, is intended to emulate both the nutrients, and chemical inhibitors of microbial growth, present in various styles of cheese. All broths were inoculated with cultures of *Listeria* spp. (*L. monocytogenes* or *L. innocua*) or *E. coli* (R31 or M23) and incubated at 10, 15 and 20°C, which spans the range of temperature used for cheese maturation (typically in the range 12 - 16°C). *E. coli* R31 is a clinical isolate and is verotoxigenic. *E. coli* M23 is non-pathogenic strain. Pathogen survival was assessed as a function of time.

Table 2.1. Physico-chemical conditions in nutrient-rich ("milk-based medium") broths intended to emulate cheese.

Category	pH	Water activity (a_w)	L-lactic acid (g/100g)	Undissociated lactic acid (mM)
Brined cheese	4.37	0.966	0.59	15.5
Cheddar cheese	5.30	0.943	0.73	2.9
Hard grating cheese	5.40	0.890	0.68	2.1
Mould-ripened cheese	6.24	0.930	0.62	0.3
Semi-hard cheese	5.46	0.963	1.11	3.0

On the basis of the chemical parameters for all test conditions, none of the challenge organisms would be expected to grow. This is because the conditions are beyond the a_w and pH limits for growth of those organisms based on the scientific literature and predictive models for microbial growth or growth limits (ICMSF, 1996; Presser *et al.*, 1998; Ross *et al*., 2000; Tienungoon *et al*., 2000). Specifically, the pH and undissociated lactic acid concentration of brined cheese are beyond the growth range of bacteria. Similarly, the hard grating cheese has very low water activity and is sufficient to prevent most bacterial growth, but certaintly of the challenge strains. Other cheese types emulated include a combination of parameters that are sufficient, of themselves to preclude the growth of bacteria.

In addition, a series of broths, representative of the physico-chemical conditions of soft surface-ripened cheeses (i.e., pH 6.5, a_w 0.982, undissociated lactic acid 0.02 mM) was prepared and inoculated but growth of all challenge organisms was observed within a few days at all tempeatures tested. We intepret this to mean that the high pH, high water activity and low lactic acid concentrations typical of soft surface-ripened cheeses do not present hurdles to growth of *E. coli* and *Listeria* at temperatures ≥10°C. The scientific literature also attests to the potential for growth of *L. monocytogenes* in soft, surface-ripened cheeses as did a 2012-2013 outbreak of listeriosis in Australia linked to this style of cheese from a single manufacturer (VicDOH, 2013).

2.3 Results

Figures 2.1a-e, and Figures 2.2a-e show the inactivation responses of *Listeria* spp. and *E. coli* in broths intended to emulate different types of cheese. In all cases except the simulated brined cheese broth, a biphasic inactivation reponse was observed for both *Listeria* spp. and *E. coli*. There was a delay of up to 80 days before inactivation of *Listeria* spp. was observed. By contrast, *E. coli* appeared to have an initial rapid phase of inactivation followed by a much longer second, slower, phase of inactivation.

Inactivation rates for each species in each type of test broth were calculated by linear regression. In the case of *Listeria* spp., rates in the second, faster, phase of inactivation were reported. However, data on the time before inactivation commenced was also estimated because prediction of inactivation based on rate only would severely overestimate the extent of pathogen inactivation over time and could lead to public health risk. In the case of *E. coli*, rates in the second, slower, phase of inactivation were recorded and collated. This approach will lead to prediction of less inactivation than would probably occur, because the initial rapid phase of inactivation is ignored. Inactivation rate data for each organism, temperature and cheese-broth are detailed in Appendix 3.

Arrhenius plots were generated and used to describe inactivation rates as a function of temperature for each strain of each species/genus, as shown in Figure 2.3. The average rate of inactivation of *E. coli* in fermented meats and related broths, as determined by McQuestin *et al.* (2009) is also shown for comparison, to assess the hypothesis that non-thermal inactivation kinetics are the same, irrespective of growth-preventing conditions, genus/species of organism, or media in which bacteria were inoculated (Zhang *et al.*, 2010).

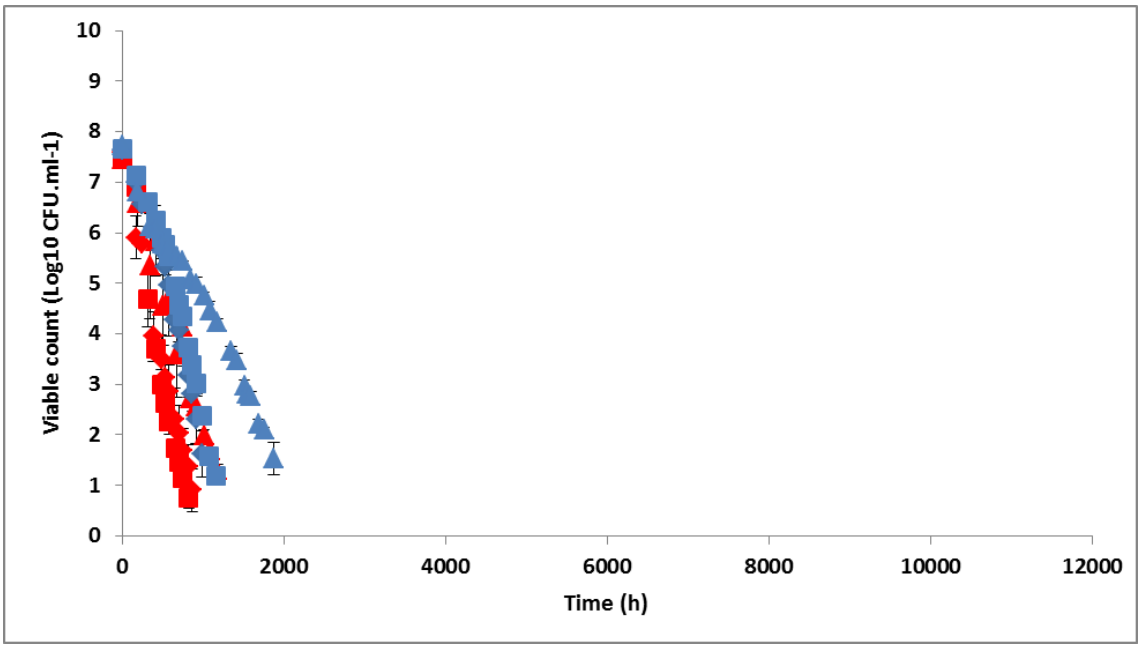


Figure 2.1a. Inactivation data for *Listeria* spp. in brined cheese-like broth. *L. monocytogenes* Scott A incubated at 10°C (▲), 15°C (■) and 20°C (◆); and *L. innocua* at 10°C (▲), 15°C (■) and 20°C (◆).

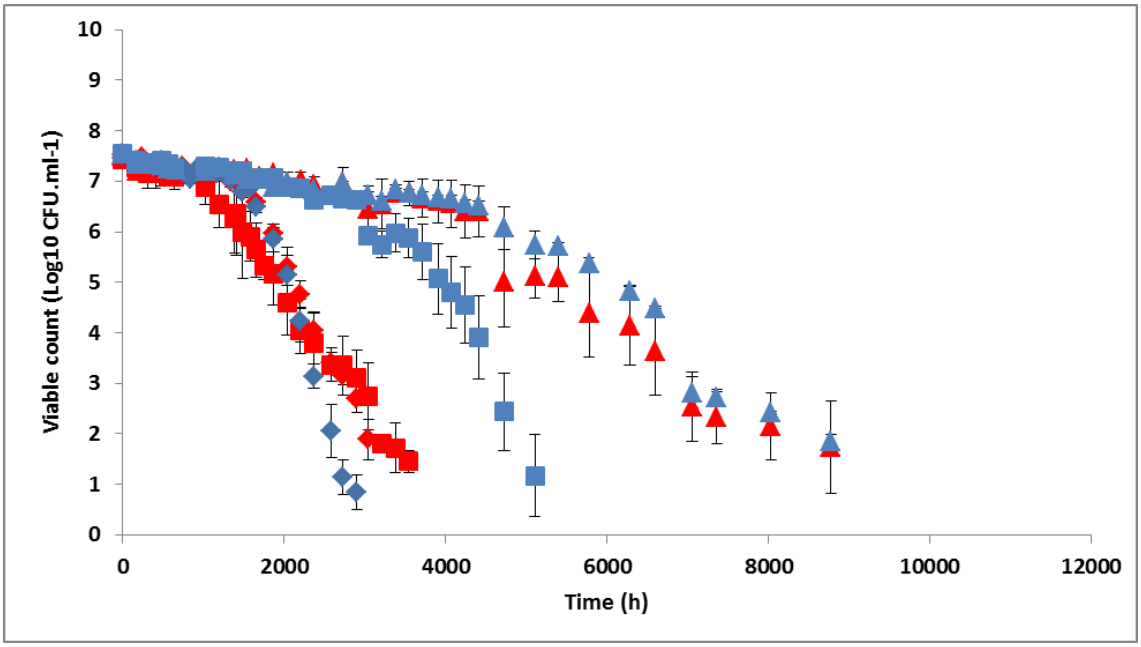


Figure 2.1b. Inactivation data for *Listeria* spp. in Cheddar cheese-like broth. *L. monocytogenes* Scott A incubated at 10°C (▲), 15°C (■) and 20°C (◆); and *L. innocua* at 10°C (▲), 15°C (■) and 20°C (◆).

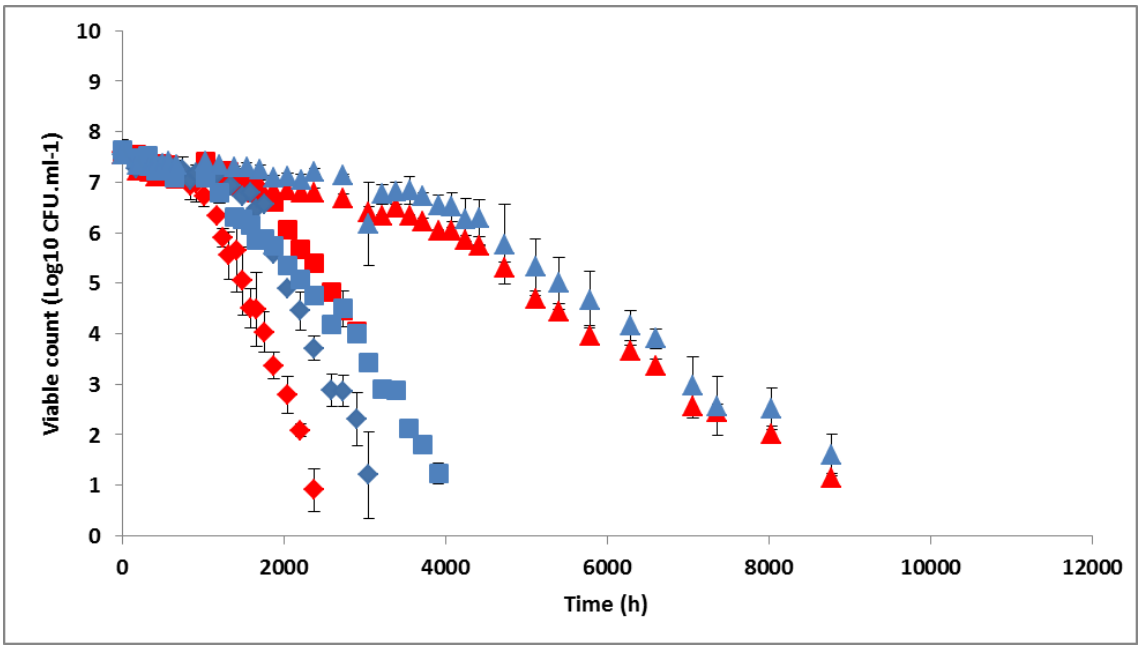


Figure 2.1c. Inactivation data for *Listeria* spp. in hard-grating cheese-like broth. *L. monocytogenes* Scott A incubated at 10°C (▲), 15°C (■) and 20°C (◆); and *L. innocua* at 10°C (▲), 15°C (■) and 20°C (◆).

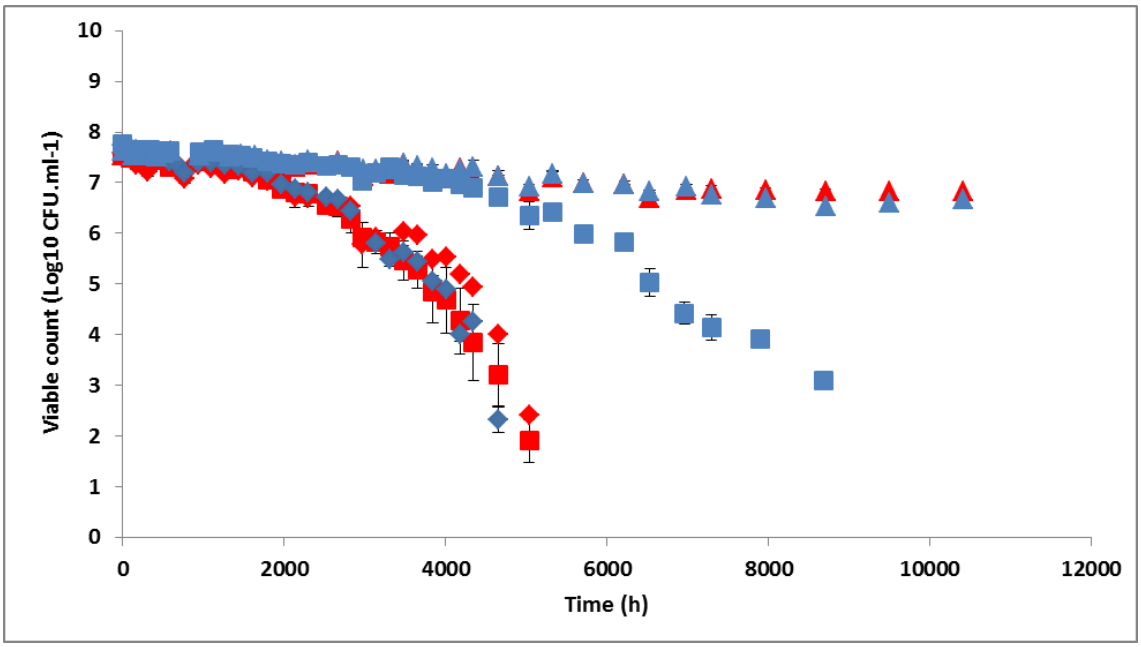


Figure 2.1d. Inactivation data for *Listeria* spp. in mould-ripened cheese-like broth. *L. monocytogenes* Scott A incubated at 10°C (▲), 15°C (■) and 20°C (◆); and *L. innocua* at 10°C (▲), 15°C (■) and 20°C (◆).

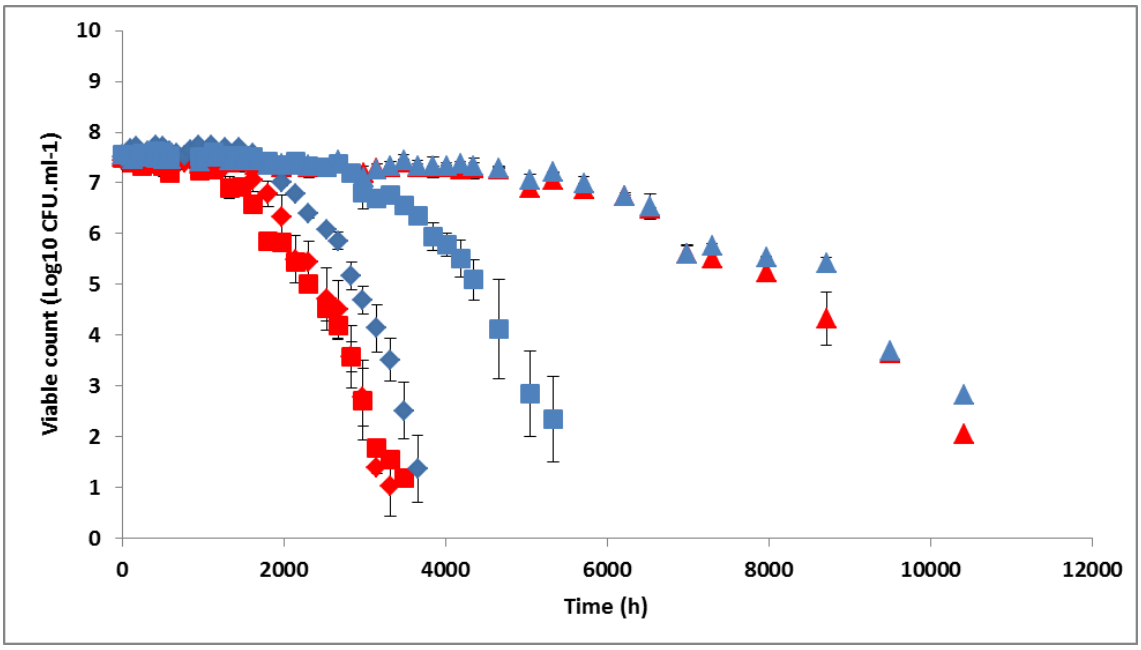


Figure 2.1e. Inactivation data for *Listeria* spp. in semi-hard cheese-like broth. *L. monocytogenes* Scott A incubated at 10°C (▲), 15°C (■) and 20°C (◆); and *L. innocua* at 10°C (▲), 15°C (■) and 20°C (◆).

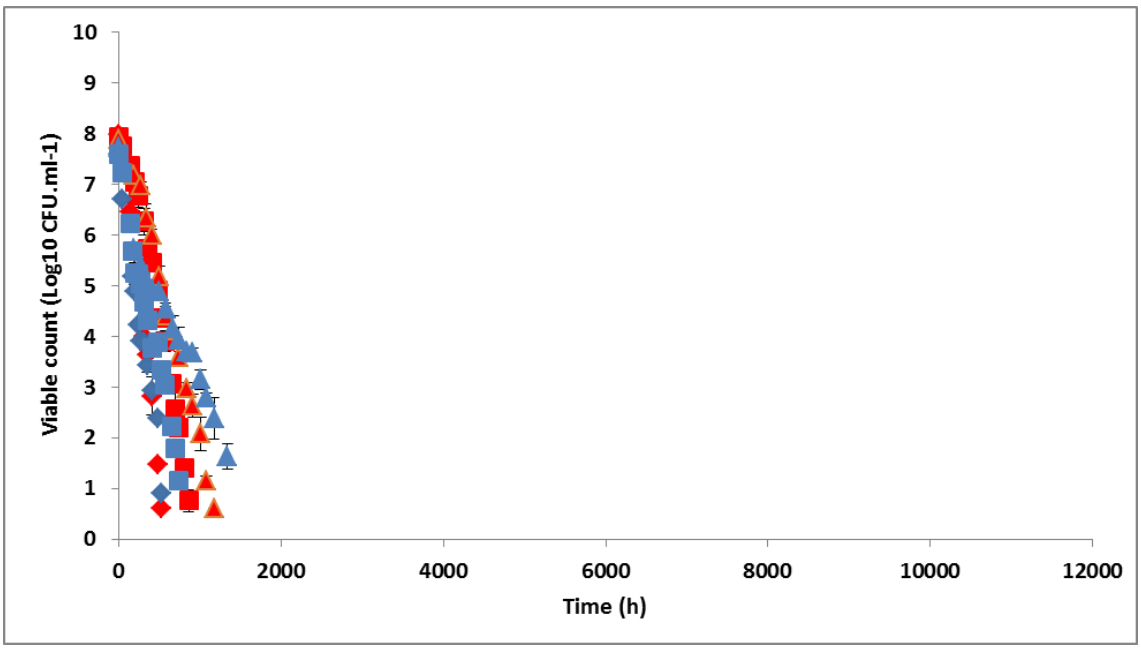


Figure 2.2a. Inactivation data for *E. coli* in brined cheese-like broth. *E. coli* R31 incubated at 10°C (▲), 15°C (■) and 20°C (◆); and *E. coli* M23 at 10°C (▲), 15°C (■) and 20°C (◆).

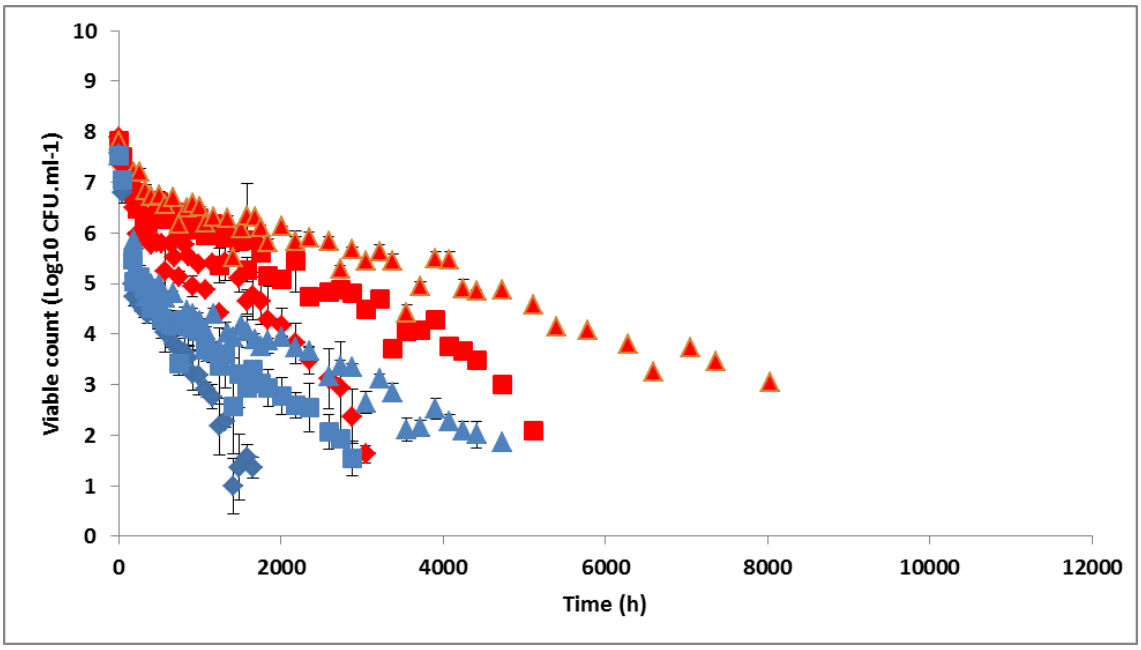


Figure 2.2b. Inactivation data for *E. coli* in Cheddar cheese-like broth. *E. coli* R31 incubated at 10°C (▲), 15°C (■) and 20°C (◆); and *E. coli* M23 at 10°C (▲), 15°C (■) and 20°C (◆).

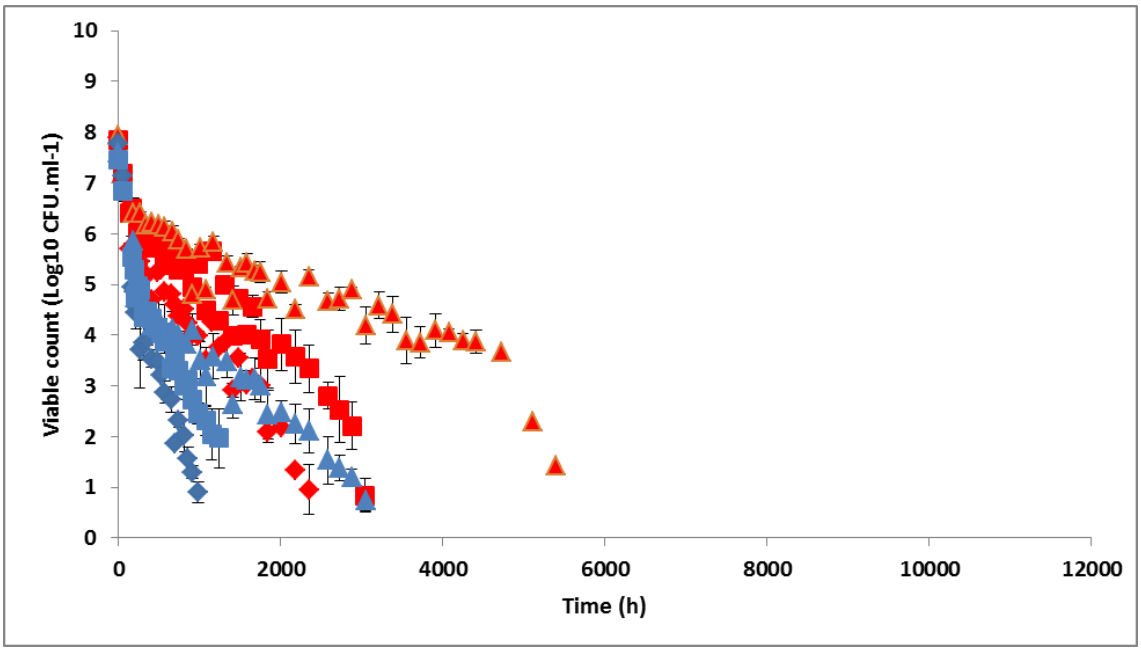


Figure 2.2c. Inactivation data for *E. coli* in hard-grating cheese-like broth. *E. coli* R31 incubated at 10°C (▲), 15°C (■) and 20°C (◆); and *E. coli* M23 at 10°C (▲), 15°C (■) and 20°C (◆).

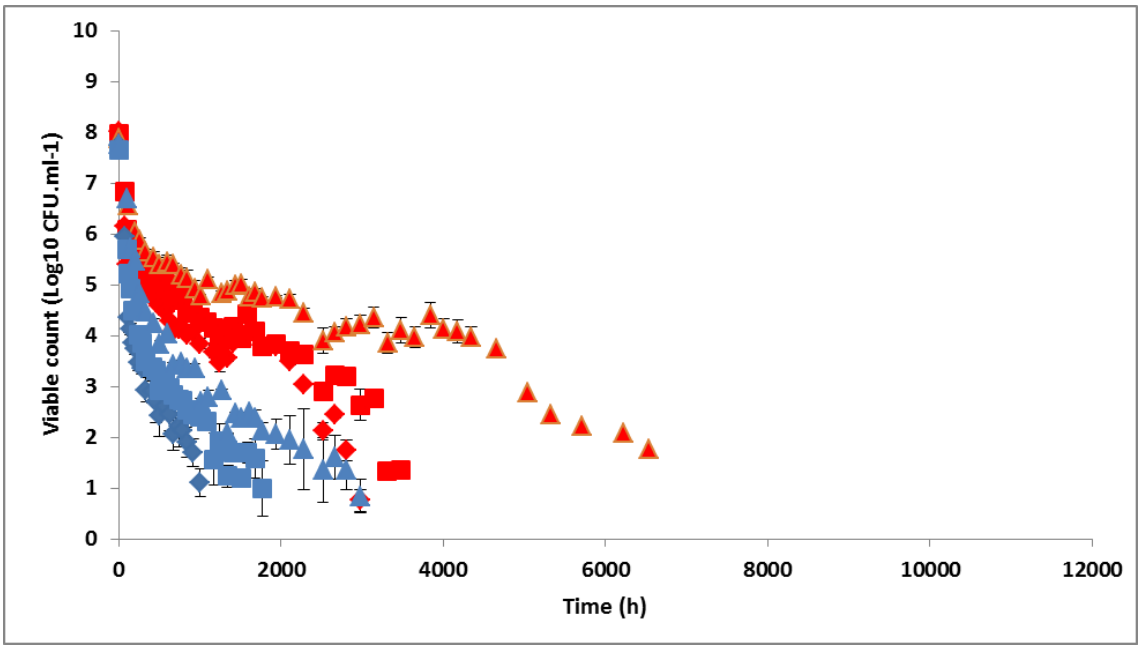


Figure 2.2d. Inactivation data for *E. coli* in mould-ripened cheese-like broth. *E. coli* R31 incubated at 10°C (▲), 15°C (■) and 20°C (◆); and *E. coli* M23 at 10°C (▲), 15°C (■) and 20°C (◆).

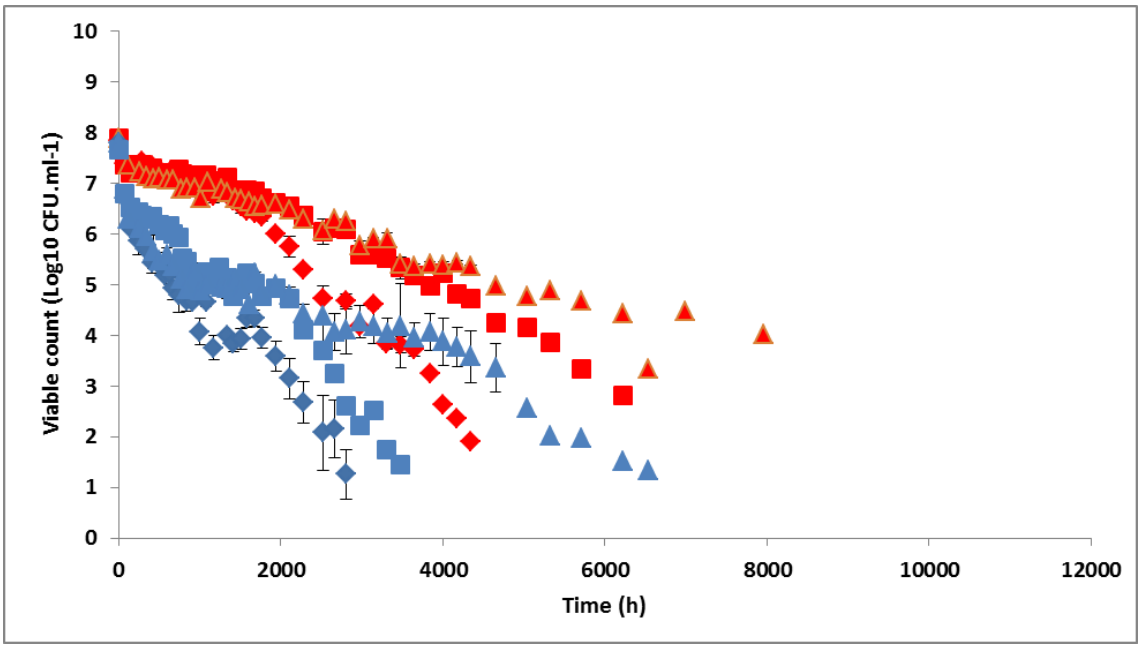


Figure 2.2e. Inactivation data for *E. coli* in semi-hard cheese-like broth. *E. coli* R31 incubated at 10°C (▲), 15°C (■) and 20°C (◆); and *E. coli* M23 at 10°C (▲), 15°C (■) and 20°C (◆).

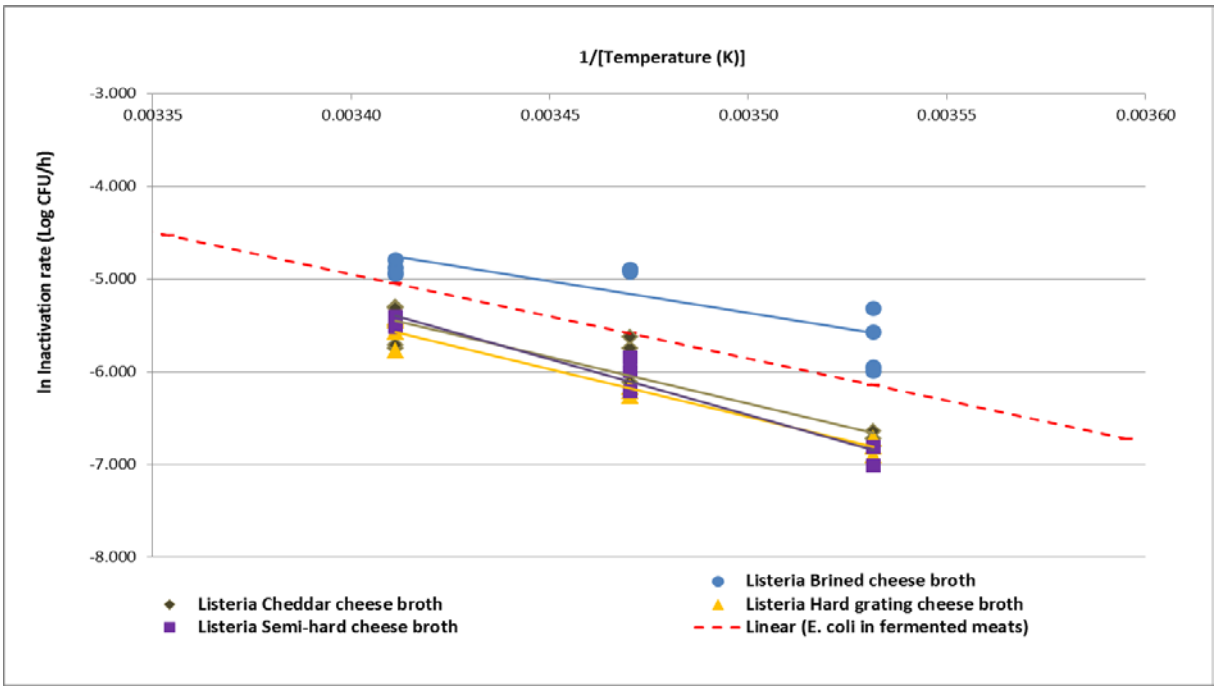


Figure 2.3a. Arrhenius plot of inactivation rates for *Listeria* spp. in simulated cheese broths compared to inactivation rates for *E. coli* in fermented meats. The red-dashed line is the model of McQuestin *et al.* (2009) for the inactivation rates of *E. coli* in fermented meats.

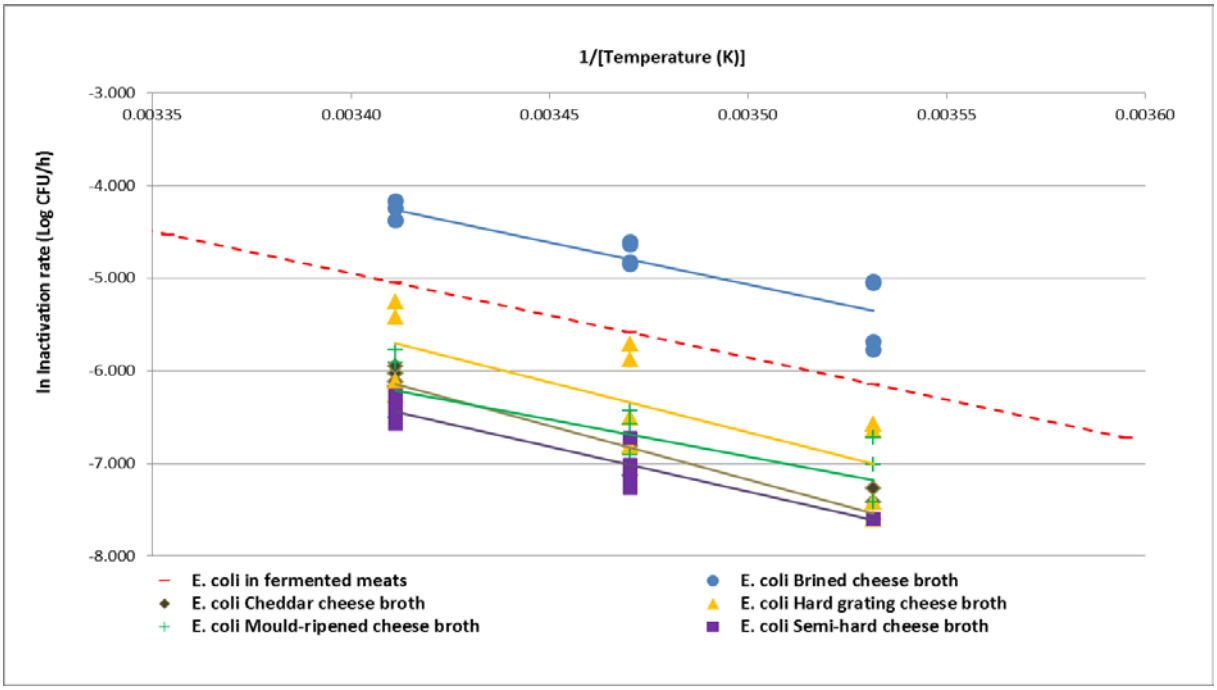


Figure 2.3b. Arrhenius plot of inactivation rates for *E. coli* in simulated cheese broths compared to inactivation rates for *E. coli* in fermented meats. The red-dashed line is the model of McQuestin *et al.* (2009) for the inactivation rates of *E. coli* in fermented meats.

As discussed earlier, the lag time observed before commencement of rapid inactivation of *Listeria* spp. is an important consideration from a public health perspective and needs to be factored into any model to predictive the rates of inactivation of Listeria in cheeses. Accordingly, the times before commencement of inactivation at each temperature were estimated from Figures 2.1b - e. The inverse of those times, which can be interpreted as *rates* of lag phase depletion, were then presented as an Arrhenius plot as shown in Figure 2.4. It is apparent that the reciprocal of time taken before inactivation commences responds to temperature in the same manner as does inactivation rate. It is notable that the time to inactivation is slower in the broths that emulate mould-ripened cheese. That broth has relatively high pH and water activity neither of which, acting alone, would prevent growth of *L. monocytogenes*, or *innocua*. On the other hand, there was no lag time in the very harsh environment of the brined cheese-broth. This suggests that the survival time prior to commencement of inactivation may be related to the stress that the cheese environment imposes on cells that may be present in the cheese or milk used to make it.

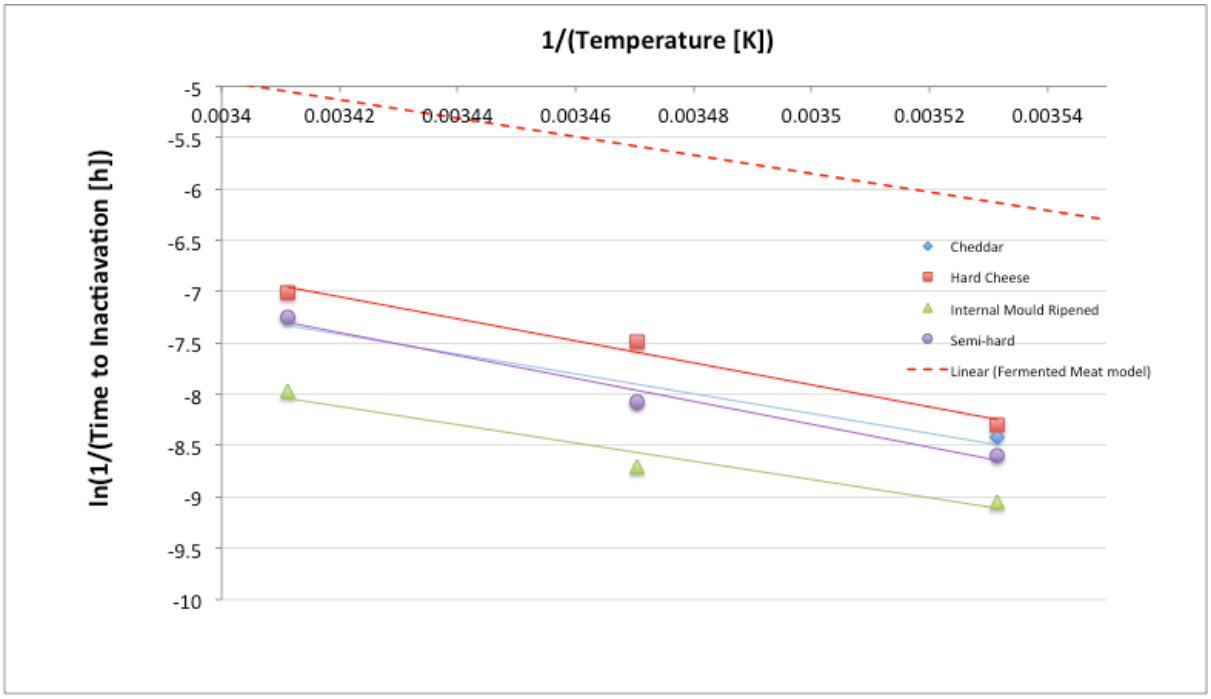


Figure 2.4. Arrhenius plot of rates before commencement of inactivation for *Listeria* spp. in simulated cheese broths. In the figure the reciprocal of the time to commencement of inactivation of *Listeria* species in various cheese broths (*see* Figures 2.1b-e) was calculated, and treated as the 'rate' of resolution of the lag time before inactivation is evident. The red-dashed line is the model of McQuestin *et al.* (2009) for the *inactivation rate* of *E. coli* in fermented meats.

2.4 Conclusions

Figures 2.3a, b show that the previously observed effect of temperature on the rates of inactivation of *Listeria* spp. and *E. coli* is preserved in cheese-like broths. For both sets of challenge organisms

temperature had a very similar effect on the relative rate of inactivation. This implies that the relative effect of temperature on inactivation of bacteria may be independent of species. It was also evident that the temperature dependence of relative inactivation rates of *Listeria* spp. and *E. coli* is consistent with earlier work (McQuestin *et al.*, 2009), suggesting that the effects of temperature on the inactivation of bacteria is not dependent upon the types of foods and/or media in which they are inoculated, provided that those foods preclude growth of those pathogens.

However, the data presented reveal that inactivation rates of *Listeria* spp. and *E. coli* in brined-cheese-like broths were faster than average inactivation rates in fermented meats and rates in other cheese-like broths. The basis of these differences is not known, but it is apparent that brined cheeses typically have a lower pH and much higher undissociated lactic acid concentrations than other cheese styles and in the broths used in this study to emulate them (Table 2.1). This suggests that the inactivation response of bacteria in foods might not only be affected by temperature, but also by pH and/or undissociated organic acid levels if the levels are severe enough.

Systematic differences in the responses of *E. coli* strains to the stresses applied were evident, in the initial rapid phase of inactivation, with *E. coli* M23 being more sensitive to the conditions and typically showing a 2 - 3 $\log_{10}$CFU greater inactivation during the first, rapid, phase of inactivation. In the second slower phase of inactivation the rates of inactivation of both *E. coli* strains were apparently more similar but the inactivation rate of *E. coli* M23 was also faster, typically by a factor of 1.5 to 2. In this situation the use of the slower inactivation rate estimates, characteristic of the second phase, will generate a conservative ("fail-safe") model. Interestingly, differences in absolute inactivation rates between the two species of *Listeria* investigated are less than between the two strains of *E. coli*. However, the physiological differences between *L. innocua* and *L. monocytogenes* are very small, perhaps only due to a score of genes, whereas the genetic differences between verotoxigenic and non-pathogenic strains of *E. coli* may be much greater.

Figure 2.4 shows that that the more complex pattern of inactivation observed for the *Listeria* spp. is also reproducible, and suggests that the initial lag period before commencement of the second, rapid inactivation phase, also responds to temperature qualitatively as does the inactivation rate. However, differences between broths that could be related to the harshness of the environment may also need to be considered in the development of predictive models for *Listeria* inactivation in cheeses.

It should also be emphasised, however, that the times prior to commencement of inactivation of *Listeria* spp. at 15°C were typically in the range 1500 - 2000 hours, or approximately 60 - 80 days. Many cheeses are considered to be ready for sale within this time and, accordingly, cheese containing *Listeria monocytogenes* could be released for sale before any significant inactivation of the pathogen had occurred. This is of particular concern for surface and internal mould-ripened cheeses, which typically have maturation times of two to four months prior to release for sale.

3 Evaluation of the broth based model for generation of inactivation data relevant to pathogens in mature cheese

3.1 Introduction

Earlier observations in broths intended to emulate cheeses showed biphasic inactivation kinetics of both *Listeria* spp. and *E. coli* (*see* Section 2). Long period of stasis (i.e., no inactivation) were observed for *L. monocytogenes* and *L. innocua* in simulated cheese broths, whereas a short period of rapid inactivation followed by a slower, more prolonged, but constant rate of inactivation was observed in many experiments involving *E. coli*. These are in contrast to previous studies on *E. coli* inactivation in fermented meats and analogous broths (Ross *et al*., 2001; Ross *et al*., 2004). It was evident in those studies that *E. coli* displayed a more-or-less constant rate of inactivation once conditions in the model broth-based system that simulates salami batter became inimical to *E. coli* growth, and similar results were observed in experimental salamis. As a consequence, this has led to concern that inactivation of target organisms in cheese might differ from that observed in simulated cheese broths. To address this, we conducted a preliminary study that involved inoculating *Listeria* spp. and *E. coli* into commercial Cheddar cheese slices purchased at retail and monitoring their inactivation over time.

3.2 Approaches

Broth cultures of the two *Listeria* species and two *E. coli* strains previously described were used to inoculate slices of cheese purchased as prepacked sliced cheese at retail. After inoculating multiple 10 µL spots between two slices, the two slices were repacked, sandwich-like, under vacuum and replicate sets incubated at 10°C, 20°C or 25°C. Duplicate samples were removed periodically and processed to determine the numbers of the challenge organisms remaining. The temperatures chosen do not represent normal storage practice, but are relevant to normal handling of cheeses. Upper temperatures were chosen so as to be able to accelerate this study and the availability of results.

3.3 Observations

Figures 3.1a, b show the same distinct patterns of inactivation of both *Listeria* spp. and *E. coli* in cheeses as observed in cheese-like broths (*see* Section 2). Specifically, *Listeria* spp. showed a lag before a phase of more rapid inactivation, whereas *E. coli* seemed to display faster initial inactivation followed by a slower, second phase. Despite that this preliminary study was not continued for a long period of time (due to the limited numbers of cheese samples), the observations are consistent with earlier trials in simulated cheese broths. This suggests that broth based models could be used to generate inactivation kinetics data that are representative of bacteria in cheeses and used to generate predictive models for regulatory decision making.

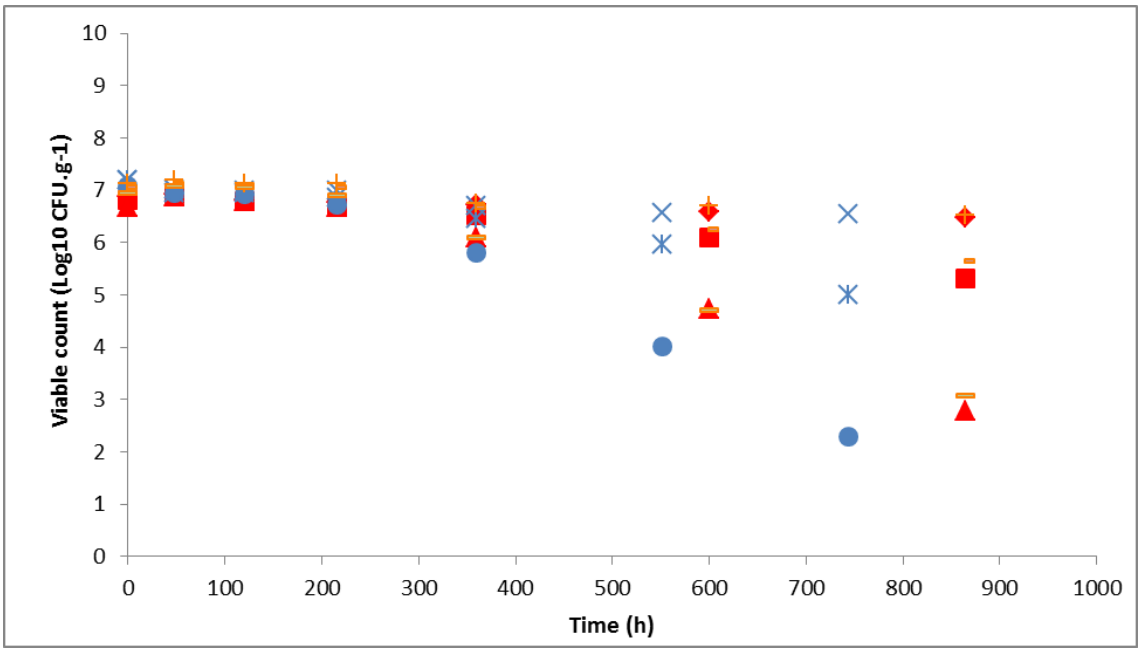


Figure 3.1a. Inactivation kinetics of *Listeria* spp. inoculated onto Cheddar cheese and stored at various temperatures. *L. monocytogenes* Scott A incubated at 10°C (◆), 20°C (■) and 25°C (▲); *L. innocua* at 10°C (×), 20°C (✳) and 25°C (●); and *L. monocytogenes* FW04/0025 at 10°C (+), 20°C (–) and 25°C (▬).

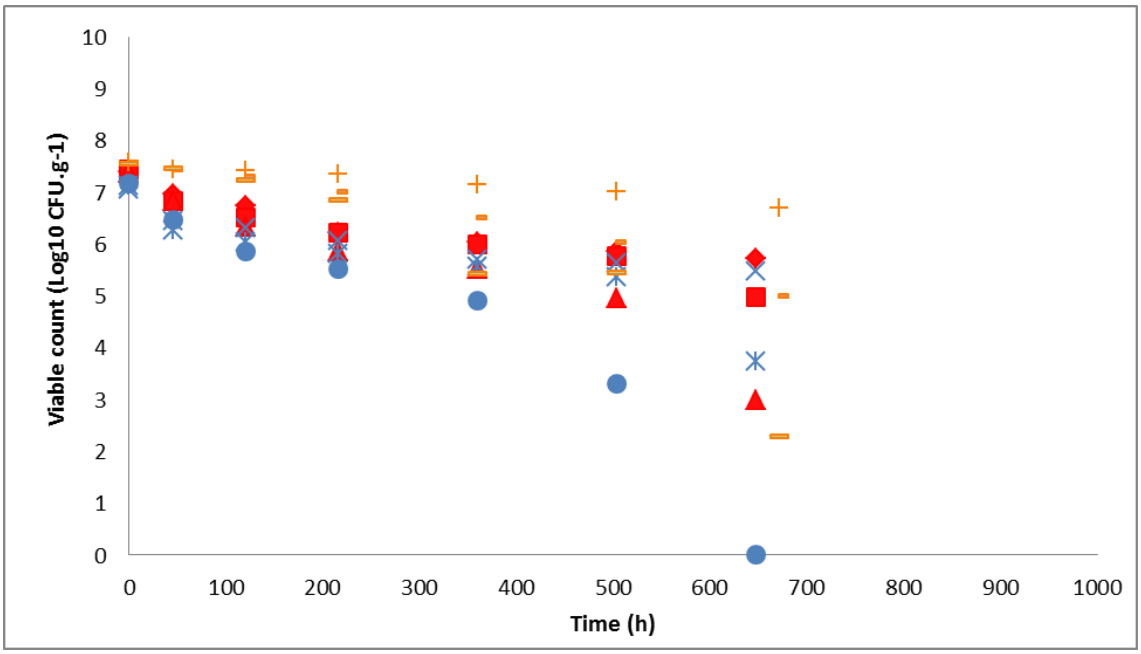


Figure 3.1b. Inactivation kinetics of *E. coli* inoculated onto Cheddar cheese and stored at various temperatures. *E. coli* K12 incubated at 10°C (◆), 20°C (■) and 25°C (▲); *E. coli* M23 at 10°C (×), 20°C (✳) and 25°C (●); and *E. coli* R31 at 10°C (+), 20°C (–) and 25°C (▬).

To evaluate further the potential of the simplified broth system to generate data for models to predict inactivation of bacteria in cheeses, another study was undertaken to characterise and compare inactivation kinetics of *Listeria* spp. and *E. coli* in Gouda cheese to those designed to simulate the same cheese. The study was conducted at three different temperatures (10, 20 and 25°C).

Figures 3.2a and 3.2b illustrate inactivation kinetics of *Listeria* spp. in Gouda cheese and Gouda cheese-like broth, respectively; kinetics of *E. coli* inactivation in Gouda cheese and Gouda cheese-like broth are shown in Figures 3.3a, b. Both *Listeria* spp. and *E. coli* showed a biphasic inactivation response in cheese and broth intended to emulate the same cheese. This also supports earlier suggestions that the broth model system could be used to evaluate inactivation kinetics of bacteria in cheeses.

Inactivation rates for both *Listeria* spp. and *E. coli* in Gouda cheese and Gouda cheese-like broth were calculated by linear regression and compared. Because biphasic inactivation kinetics were observed, rates described and compared are based on the second phase of inactivation, whether for *Listeria* spp. or *E. coli*. Calculated inactivation rate data are shown in Appendix 4. Inactivation rates of all target organisms as a function of temperature were described by Arrhenius plots and are shown in Figure 3.4. The average rate of inactivation of *E. coli* in fermented sausages, as determined by McQuestin *et al.* (2009), was also included for comparison.

The Arrhenius plots show that, in all cases, inactivation rates of both *Listeria* spp. and *E. coli* were temperature-dependent. Furthermore, these data reveal that the temperature dependence of inactivation of *E. coli* in Gouda cheese and Gouda cheese-like broth was similar to *E. coli* inactivation in fermented meats (McQuestin *et al.*, 2009), although absolute inactivation rates differ. These data support earlier suggestions that the relative effects of temperature on the inactivation of bacteria are not dependent upon the types of foods and/or media in which they are inoculated (*see* Section 2).

3.4 Conclusions

Comparison of rates of *Listeria* inactivation in Gouda cheese and Gouda cheese-like broth suggest that inactivation of *Listeria* spp. were consistently and systematically slower in cheese than in broth designed to emulate the same cheese (Figure 3.4). The basis of this observation is not known, but it was apparent that there was a longer lag period for *Listeria* spp. in cheese than in cheese-like broth, and that inactivation in cheese did not commence within the duration of the experiments at 10°C (Figure 3.2). However, it is evident from the data presented that inactivation rates of *E. coli* were similar between cheese and cheese-like broth. These observations, taken together, suggest that the models generated from broth system could be used to predict inactivation kinetics of *E. coli* in cheeses, but may be less reliable for *Listeria* inactivation in cheese.

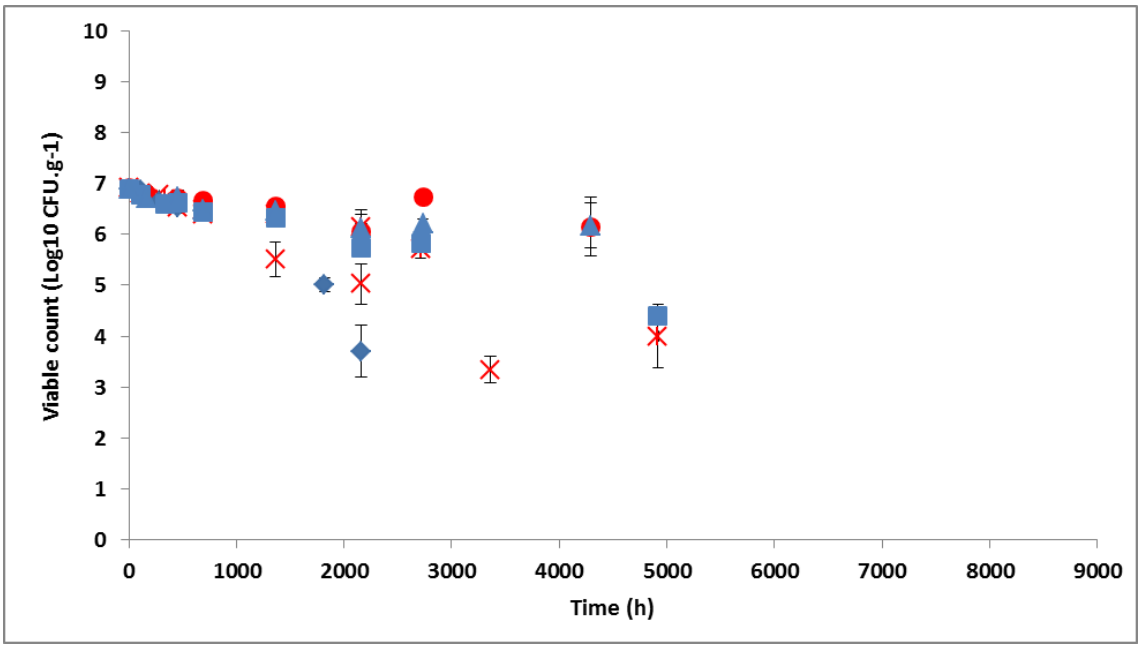


Figure 3.2a Inactivation kinetics of *Listeria* in Gouda cheese (*Listeria spp.* inoculated onto Gouda slices and re-packed under vacuum). *L. monocytogenes* Scott A incubated at 10°C (●), 20°C (✳) and 25°C (×); and *L. innocua* at 10°C (▲), 20°C (■) and 25°C (◆). Little inactivation is observed within 60 days at any temperature.

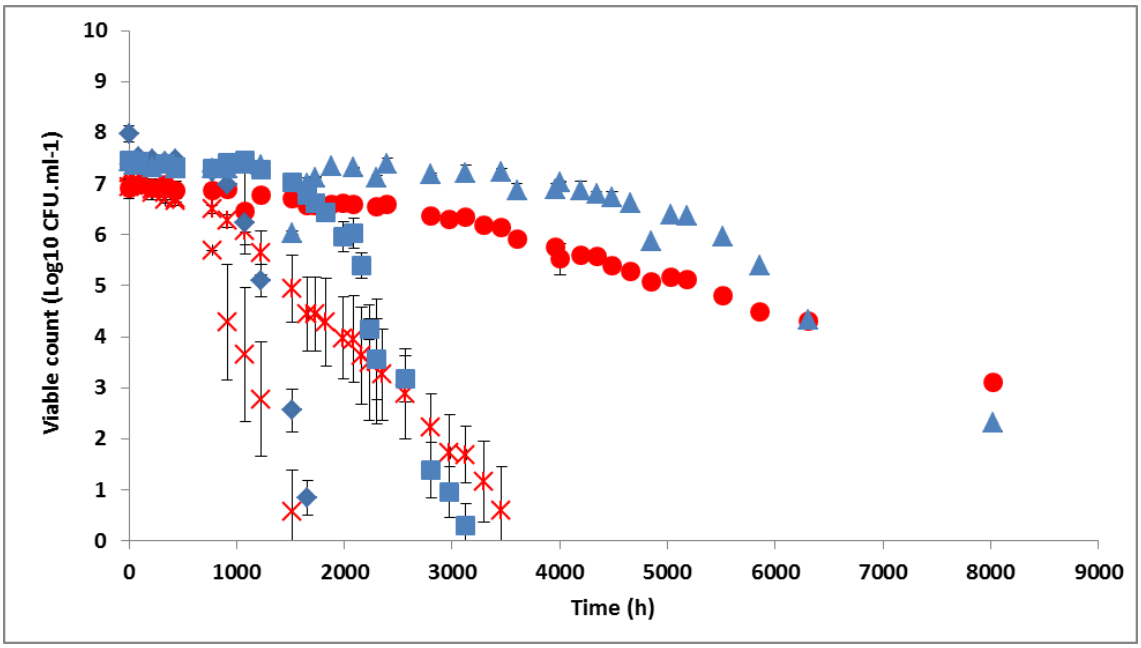


Figure 3.2b. Inactivation kinetics of *Listeria* spp. in Gouda cheese-like broth. *L. monocytogenes* Scott A incubated at 10°C (●), 20°C (✳) and 25°C (×); and *L. innocua* at 10°C (▲), 20°C (■) and 25°C (◆).

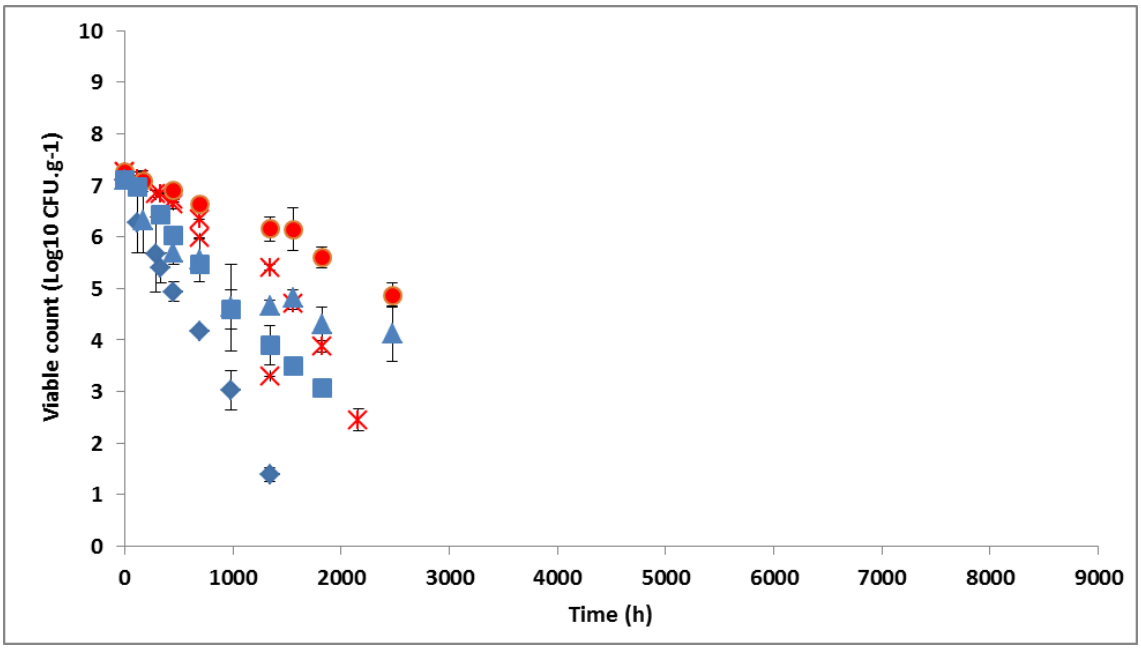


Figure 3.3a. Inactivation kinetics of *E. coli* in Gouda cheese (*E. coli* inoculated onto Gouda slices and re-packed under vacuum). *E. coli* R31 incubated at 10°C (●), 20°C (✳) and 25°C (×); and *E. coli* M23 at 10°C (▲), 20°C (■) and 25°C (◆).

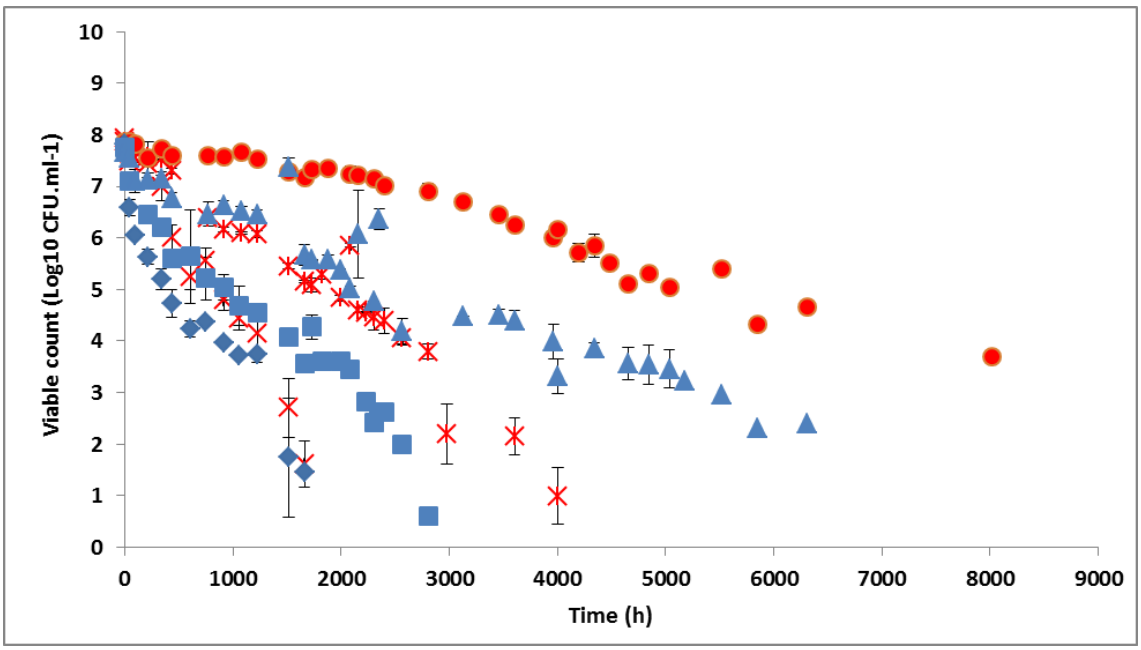


Figure 3.3b. Inactivation kinetics of *E. coli* in Gouda cheese-like broth. *E. coli* R31 incubated at 10°C (●), 20°C (✳) and 25°C (×); and *E. coli* M23 at 10°C (▲), 20°C (■) and 25°C (◆).

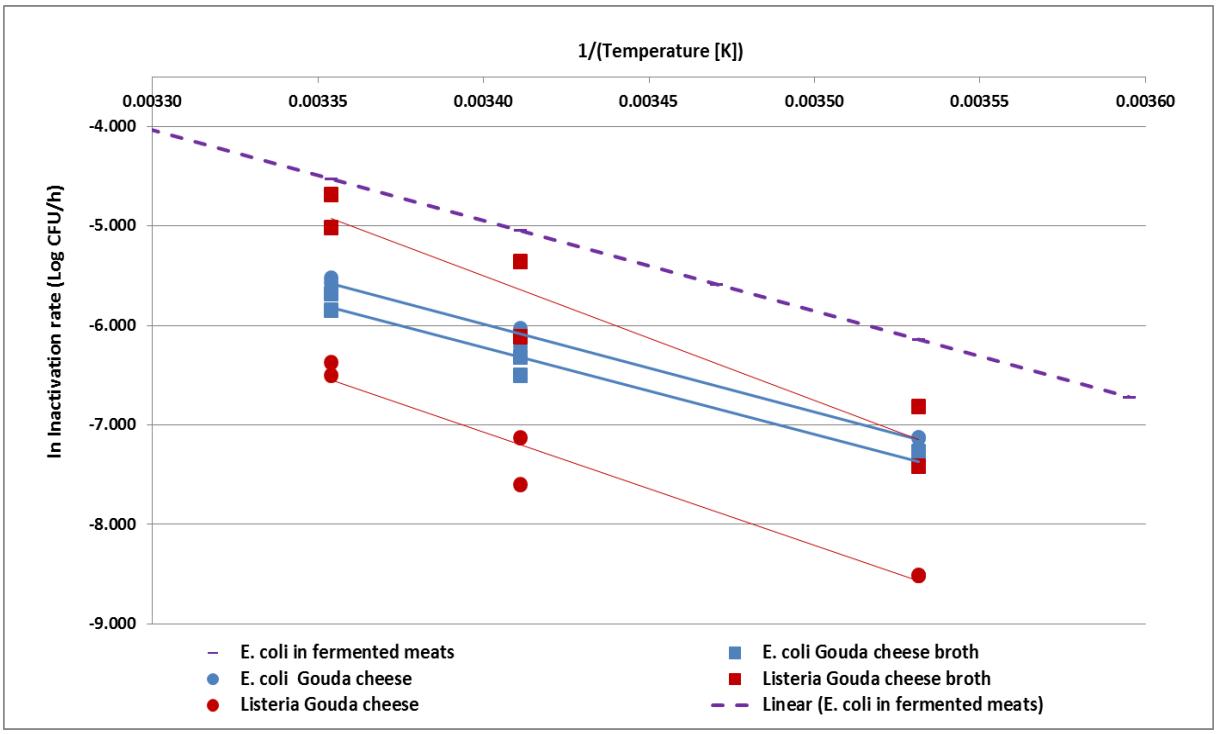


Figure 3.4. Arrhenius plot of inactivation rates for *Listeria* spp. and *E. coli* in Gouda cheese and Gouda cheese-like broths compared to inactivation rates for *E. coli* in simulated cheese broths and those for *E. coli* in fermented meats. The purple-dashed line is the model of McQuestin *et al.* (2009) for the inactivation rate of *E. coli* in fermented meats.

4 Challenge studies: raw milk *vs.* pasteurised milk cheeses

4.1 Introduction

Pasteurisation renders raw milk, and foods derived from it, less likely to cause human illness (*see* e.g., NZMPI, 2014a). Nonetheless, it has been suggested by some proponents of raw milk cheese that raw milk is more inimical to growth and survival of microbial pathogens than is pasteurised milk and that, in consequence, cheese made from raw milk is "safe". The (unproven) basis for this assertion relates to the possibility that pasteurisation inactivates biochemical components of raw milk (e.g., antibodies, enzymes involved in the lacto-peroxidase system, other bacteria that compete with pathogens, etc.) that would otherwise confer a natural protection against pathogens.

As part of this project, challenge studies involving two species of pathogenic bacteria (or relevant surrogates) were undertaken to assess whether raw milk cheeses offer different levels of protection against pathogens than do the same cheeses made from pasteurised milk. The experiments undertaken also generated data to enable evaluation of the relevance of the results from the broth model studies to actual cheeses.

A series of challenge studies involving production of Wensleydale, Cheddar, Gouda, Feta (matured at two temperatures) and double-cream Brie cheeses from both raw and pasteurised milk deliberately contaminated with mixtures of *Listeria* spp. and *E. coli* strains were conducted.

4.2 Approaches

All cheeses were prepared by an experienced home cheese maker and with initial 'tips' and ongoing advice and support from a professional cheese maker, Mr. Ashley McCoy, of Wicked Cheese (Tasmania, Australia, http:// www.wickedcheese.com.au/). Wicked Cheese is a small, but successful and award-winning Tasmanian cheese producer.

Briefly, two batches of each type of cheese were prepared both from raw milk and pasteurised milk (*i.e.,* four batches in total) each of which was deliberately contaminated with a mixture of one species of *Listeria* (*L. monocytogenes* or *L. innocua*) and one strain of *E. coli* (M23 or R31). This was done so that the fate of each species/strain could be specifically determined in each batch of cheese using common selective enumeration media (i.e., PALCAM agar media for *Listeria* spp. and Eosin Methylene Blue Levine agar for *E. coli*)[1] to enable strain variability in growth and survival to be assessed.

After tempering/fermentation and curd formation/moulding/brining, the cheeses were matured at 5°C (Feta cheese), 10°C (Cheddar cheese), or 15°C (Wensleydale, Gouda, replicate samples of Feta, double-cream Brie cheeses). Enumeration of surviving challenge organisms was undertaken periodically throughout the processes of production and maturation, while monitoring physico-chemical properties such as pH, water activity, water content and organic acid concentrations.

[1] Due to the limited ability of culture media to discriminate between strains or closely related bacterial species, if two strains of *Listeria* or two strains of *Escherichia coli* were inoculated into the same batch it would not be possible to differentiate the survival of the individual strains without considerable additional labour and cost.

Based on the chemical characteristics of commercial cheese products previously tested in this project (*see* Section 1), it was expected that the combination of parameters (i.e., pH, water activity, water content, and lactic and acetic acids) of Wensleydale, Cheddar, Gouda and Feta cheeses would present sufficient hurdles to prevent growth of the target organisms during the maturation phase. This expectation was supported by predictive microbiology models for *E. coli* (Presser *et al.*, 1998) and *L. monocytogenes* (Mejlholm and Dalgaard, 2007).

Both Wensleydale and Gouda cheeses are considered to be semi-hard cheese. They have a similar range of chemical properties to those belonging to the category of 'Semi-hard' cheeses (i.e., pH 5.46, a_w 0.963 and 0.8% w/w lactic acid (~ 4 mM undissociated lactic acid)). Cheddar and Feta cheeses belong to the categories of 'Cheddar' cheeses (i.e., pH 5.30, a_w 0.943 and ~0.8% w/w lactic acid (~ 6 mM undissociated lactic acid)) and 'Brined' cheeses (i.e., pH 4.37, a_w 0.966 and ~0.9% w/w lactic acid (~40 mM undissociated lactic acid)), repectively. The probability of inactivation of both *Listeria* spp. and *E. coli* is also reinforced by the observations of earlier experiments on the response of bacteria in broths intended to emulate those cheese styles (Section 2). On the other hand, double-cream Brie cheese is assigned to the category of 'soft, surface-ripened' cheeses. This category typically includes cheeses with high pH, high water activity, and low lactic acid concentrations (e.g., pH 6.5, a_w 0.982, undissociated lactic acid 0.02 mM; *see* Table 1.2) that are within the growth range of *Listeria* spp. and *E. coli.* Both organisms, therefore, would still be expected to grow based on the validated models of Dalgaard and colleagues (Mejlholm and Dalgaard, 2007; Mejlholm *et al.*, 2010) for *L. monocytogenes* and the University of Tasmania (Presser *et al*., 1998; Ross *et al*., 2003; Mellefont *et al.,* 2003) for *E. coli.* Results presented earlier (Section 2) also demonstrated the growth of all target organisms in the broths intended to emulate soft, surface-ripened cheeses.

4.3 Results and Discussion

Background counts in the milk used were evaluated in all cases. Typical levels in raw milk were in the range 2.5 to 3.9 $\log_{10}$CFU while counts in milk after pasteurisation were typically 0.5 to 1.5 $\log_{10}$CFU lower. Figures 4.1 - 4.4 describe the behaviours of *Listeria* spp. and *E. coli* during the making of Wensleydale, Gouda, Cheddar, and Feta cheeses, respectively. Changes in the pH and water activity of these cheeses during fermentation and maturation are presented in Appendix 6, and complete data are available on the electronic media provided with this report. The final characteristics of the cheeses were similar to commercial cheeses of the same style, but no cheese matched the 'average' properties (*see* Table 1.1) of the nominal style exactly. The Feta-style cheese had a higher pH (~5) than anticipated, while the Gouda cheeses produced had lower than 'average' water activity for the style. Similarly, the Cheddar cheeses made had slightly lower than 'average' lactic acid levels. Nonetheless, the characteristics were not dissimilar to the ranges of characteristics expected from the results presented in Table 1.1.

Generally, the data reveal an increase in numbers of the challenge organisms during curd formation of all cheese styles. This trend (of increasing pathogens levels) was observed until the brining step. The observed increased concentration of challenge bacteria was not surprising, considering the conditions during curd formation are initially favourable for growth of the starter cultures (i.e., pH and lactic acid concentrations are not expected to be limiting until the water activity hurdle is added

during the brining step) and, equally importantly, due to the concentration of cells of the challenge organisms in the milk into the curd, rather than persistence in the whey (Maher *et al*., 2001).

During cheese maturation, inactivation kinetics of *Listeria* spp. and *E. coli* were observed to be different. *Listeria* spp. showed biphasic inactivation kinetics in all cheese styles (Figures 4.1-4.4). *Listeria* spp. entered a lag phase for a period of time (from 25 to 110 days) before inactivation commenced. This pattern of inactivation of *Listeria* reinforces previous observations in retail cheese products and cheese-like broths (Sections 2 and 3). By contrast, *E. coli* inactivation seems to commence almost immediately after cheese maturation commences. This is consistent with earlier studies (Sections 2 and 3), showing biphasic inactivation of *E. coli* in mature cheeses and cheese-like broths, but differs from other studies (McQuestin *et al*., 2004) with *E. coli* in fermented meats in which constant rates of inactivation were observed throughout the process once conditions become inimical to growth, despite changing water activities. In those studies, the constant inactivation rate observed was thought to be related to the slow rate of acidification and water activity reduction, allowing the challenge organisms time to physiologically adapt to the increasingly stressful environment.

Notably, the inactivation rates determined from those challenge studies is consistent with the rate of the second phase of inactivation described earlier in this report, rather than the first, more rapid, phase in the case of *E. coli* or the post-shoulder phase for *Listeria* spp. With the possible exception of Cheddar and Brie cheeses, the responses of *Listeria* spp. and *E. coli* during production and maturation of all cheese styles were similar between analogous cheeses made from raw and pasteurised milk (Figures 4.1 - 4.4). Inactivation responses of *L. monocytogenes* and *E. coli* R31 during maturation of Cheddar cheese differed between raw milk cheese and cheese made from pasteurised milk (Figures 4.3a, b) although this was not noted for the cheese inoculated with both *L. innocua* and *E. coli* M23. Notably, in these challenge trials, pathogens often survived longer in the raw milk product: inactivation of these organisms commenced much later in raw milk cheese (>110 days after cheese maturation) than in cheese made from pasteurised milk (within 50 day). The basis of these differences is not known. Furthermore, *Listeria* inactivation was different between cheese types of the same style (i.e., Wensleydale and Gouda cheeses belonging to the category of 'semi-hard' cheeses). Inactivation of *Listeria* spp. in Wensleydale cheese was not evident even more than 60 days after cheese maturation, whereas a decrease in *Listeria* numbers was observed within the first 40 days of Gouda maturation (Figs. 4.1a, b and 4.2a). This might be due to the differences in physico-chemical properties (i.e., pH, water activity and lactic acid concentrations etc.) of these two cheeses, or subtle differences in the preparation of the inoculum. Indeed, Wensleydale cheese had a higher water activity than Gouda cheese, although both cheese styles had a similar pH (ranging from 5.2 to 5.5) (see Appendix 6). It was found that Wensleydale cheese had water activity ranging from 0.961 to 0.974, whereas the water activity of Gouda cheese varied between 0.920 and 0.947. This infers that Gouda cheese would present a more hostile environment for bacteria than Wensleydale cheese.

The kinetic responses of the four challenge organisms in four different styles of cheese made from both raw and pasteurised milk are presented overleaf (Figures 4.1 – 4.4). To facilitate comparison of survival responses between different cheeses the time scale on most plots is 4500 h (~6 months), even though not all trials were followed for this amount of time, particularly if the normal processing

and maturation period was less than 6 months (e.g., Brie-style cheese, Feta-style cheese). Note also that the temperature of maturation varies according to cheese style and that higher temperatures would be expected to lead to faster inactivation.

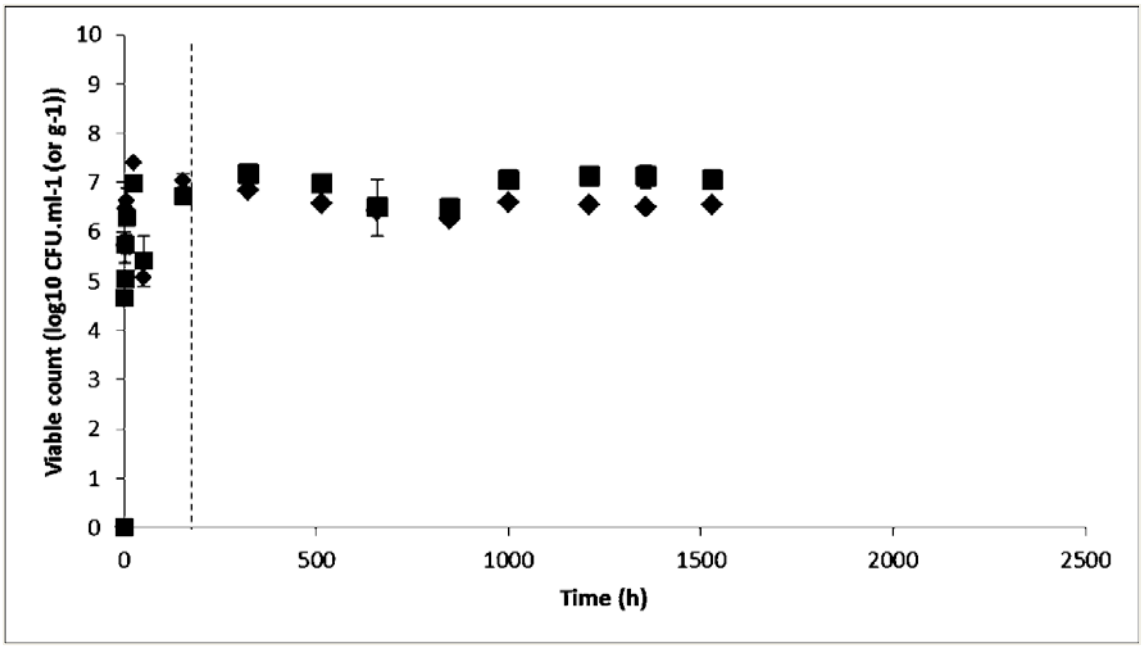


Figure 4.1a. Population changes of *L. innocua* during the making of Wensleydale-style cheeses made from raw milk (◆) and pasteurised milk (■). The dashed line differentiates the initial cheese formation (left side) from the maturation period (right side). Maturation at 15°C.

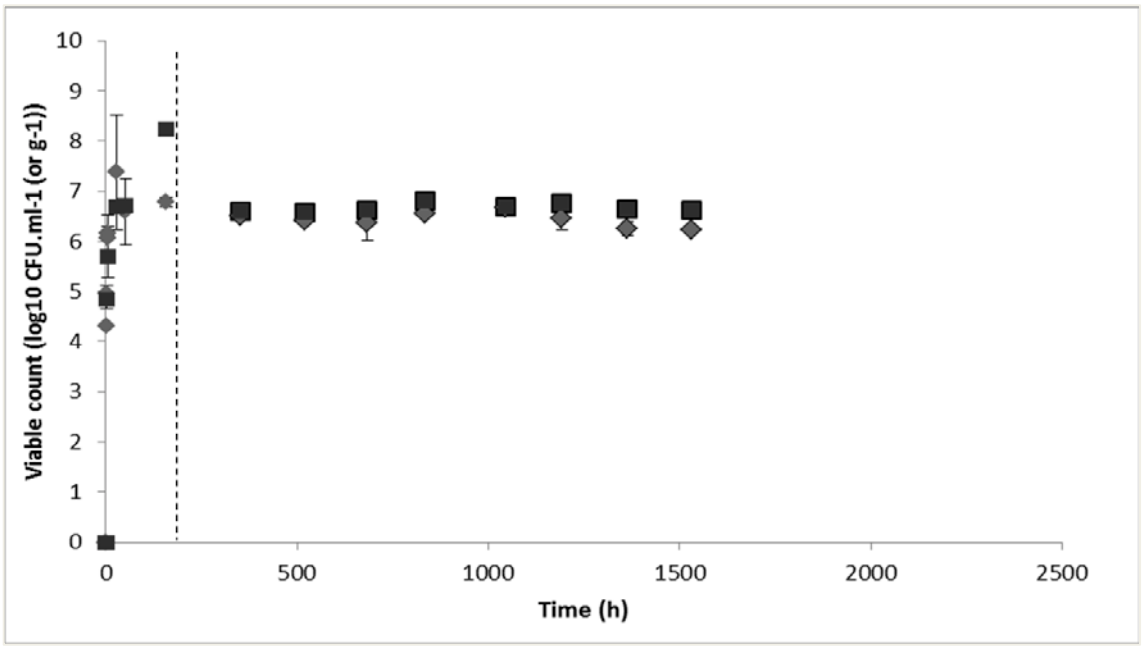


Figure 4.1b. Population changes of *L. monocytogenes* Scott A during the making of Wensleydale-style cheeses made from raw milk (◆) and pasteurised milk (■). The dashed line differentiates the initial cheese formation (left side) from the maturation period (right side). Maturation at 15°C.

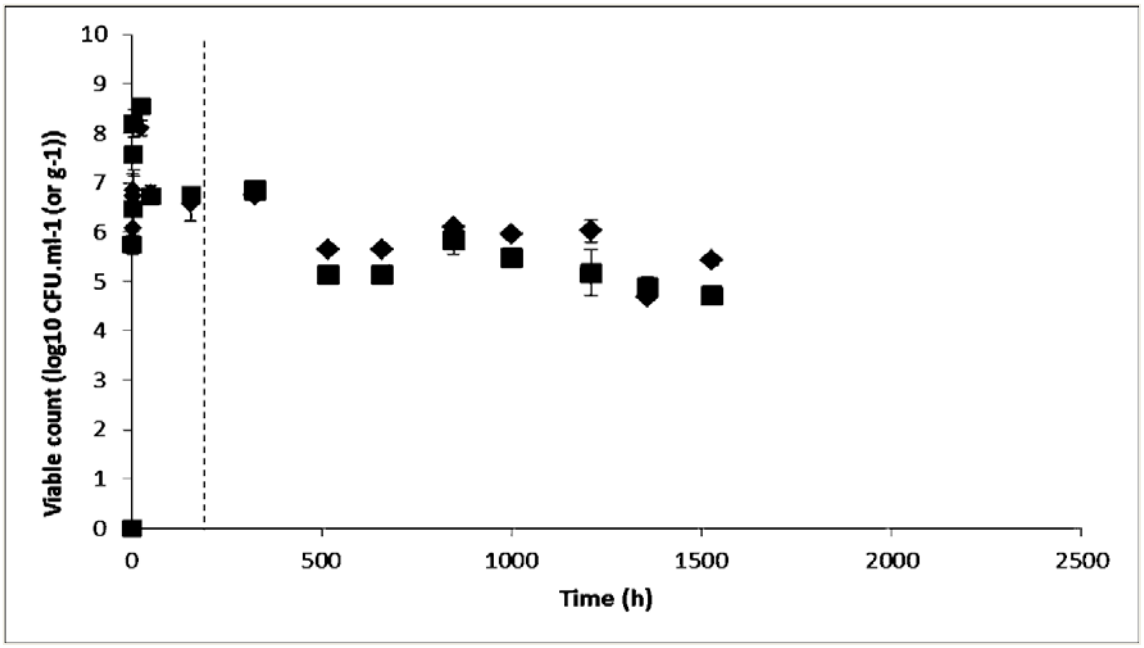


Figure 4.1c. Population changes of *E. coli* M23 during the making of Wensleydale-style cheeses made from raw milk (◆) and pasteurised milk (■). The dashed line differentiates the initial cheese formation (left side) from the maturation period (right side). Maturation at 15°C.

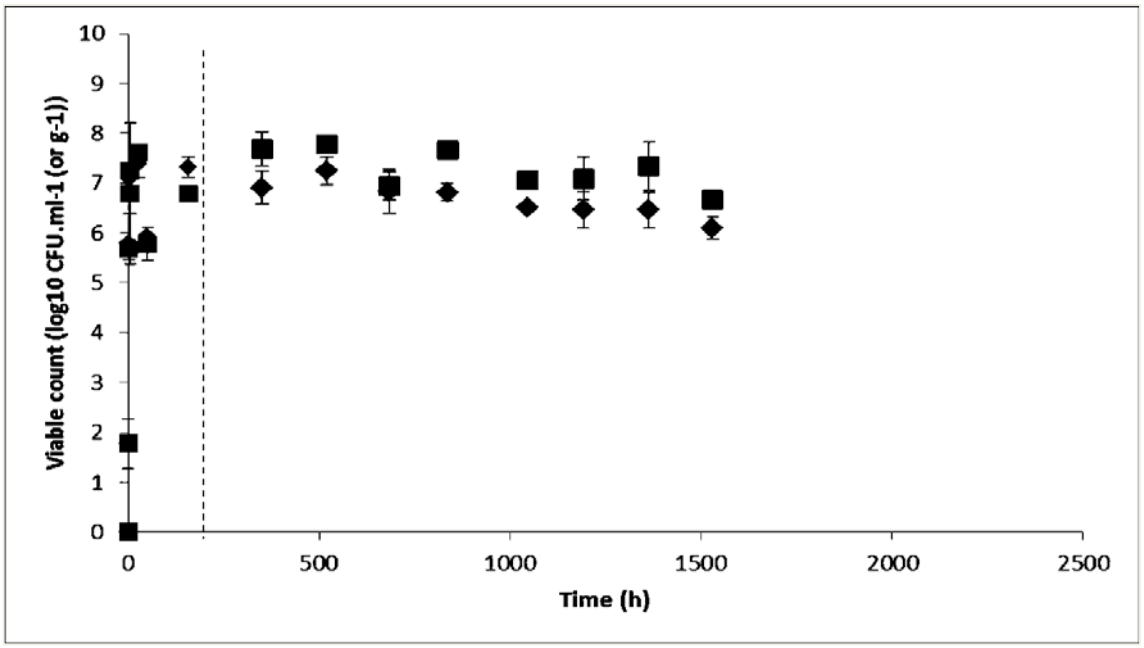


Figure 4.1d. Population changes of *E. coli* R31 during the making of Wensleydale-style cheeses made from raw milk (◆) and pasteurised milk (■). The dashed line differentiates the initial cheese formation (left side) from the maturation period (right side). Maturation at 15°C.

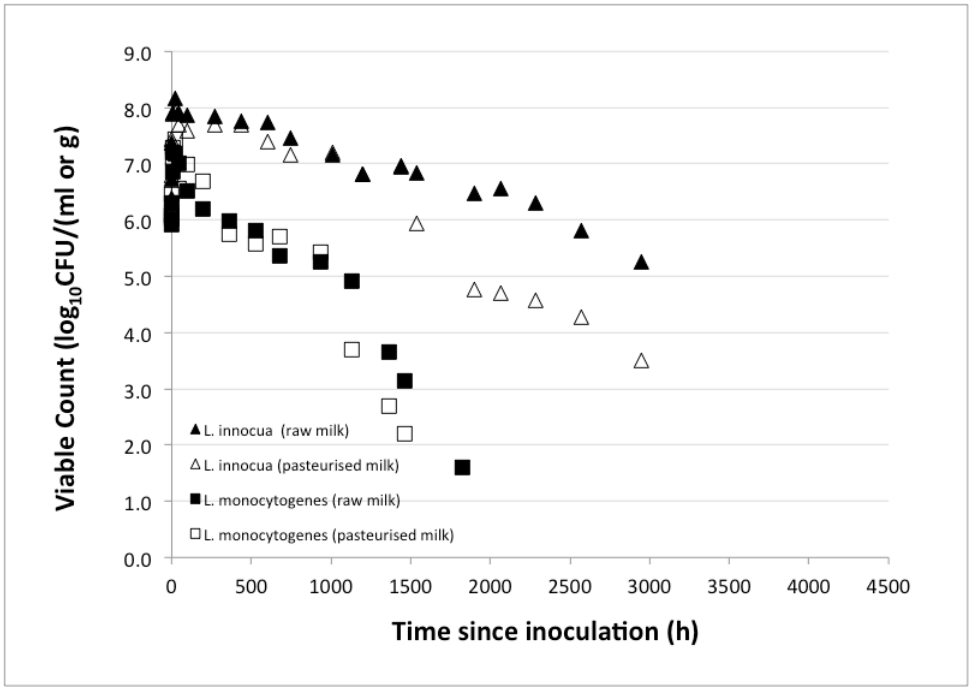


Figure 4.2a. Population changes of *L. innocua* (triangles) and *L. monocytogenes* Scott A (squares) during the making of Gouda-style cheeses made from raw milk (closed symbols) and pasteurised milk (open symbols). Maturation at 15°C.

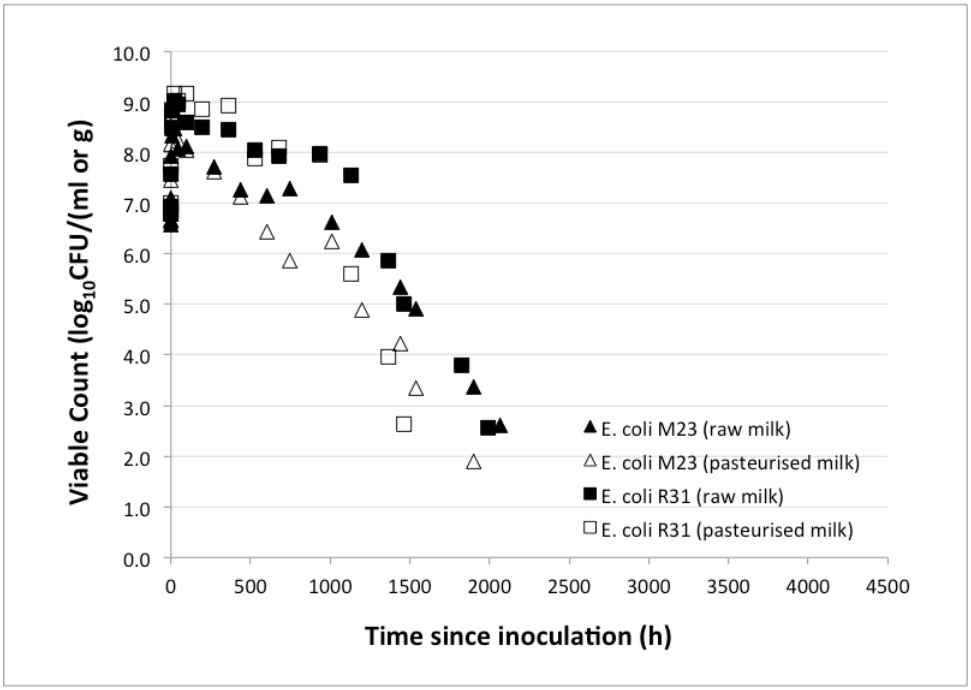


Figure 4.2b. Population changes of *E. coli* M23 (triangles) and *E. coli* R31 (square symbols) during the making of Gouda-style cheeses made from raw milk (closed symbols) and pasteurised milk (open symbols). Maturation at 15°C.

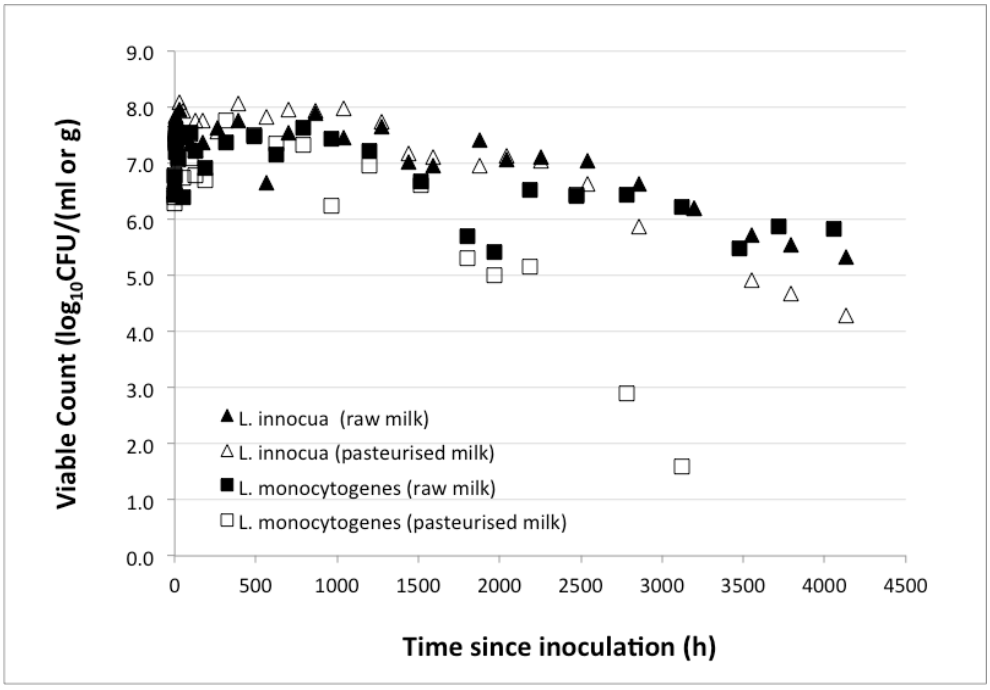


Figure 4.3a. Population changes of *L. innocua* (triangles) and *L. monocytogenes* Scott A (squares) during the making of Cheddar-style cheeses made from raw milk (closed symbols) and pasteurised milk (open symbols). Maturation at 10°C.

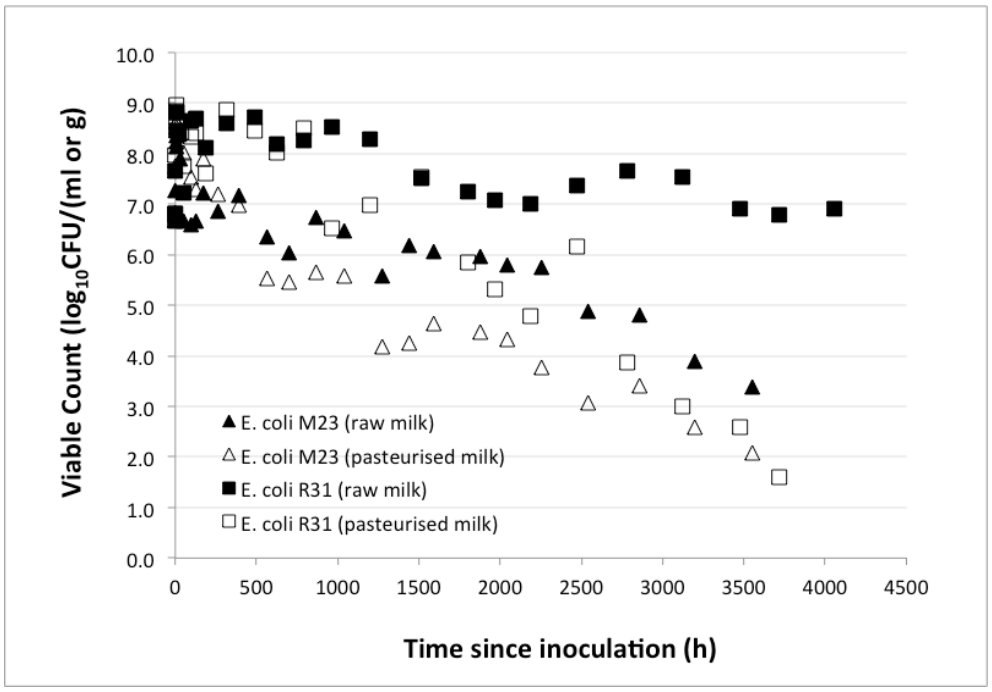


Figure 4.3b. Population changes of *E. coli* M23 (triangles) and *E. coli* R31 (squares) during the making of Cheddar-style cheeses made from raw milk (closed symbols) and pasteurised milk (open symbols). Maturation at 10°C.

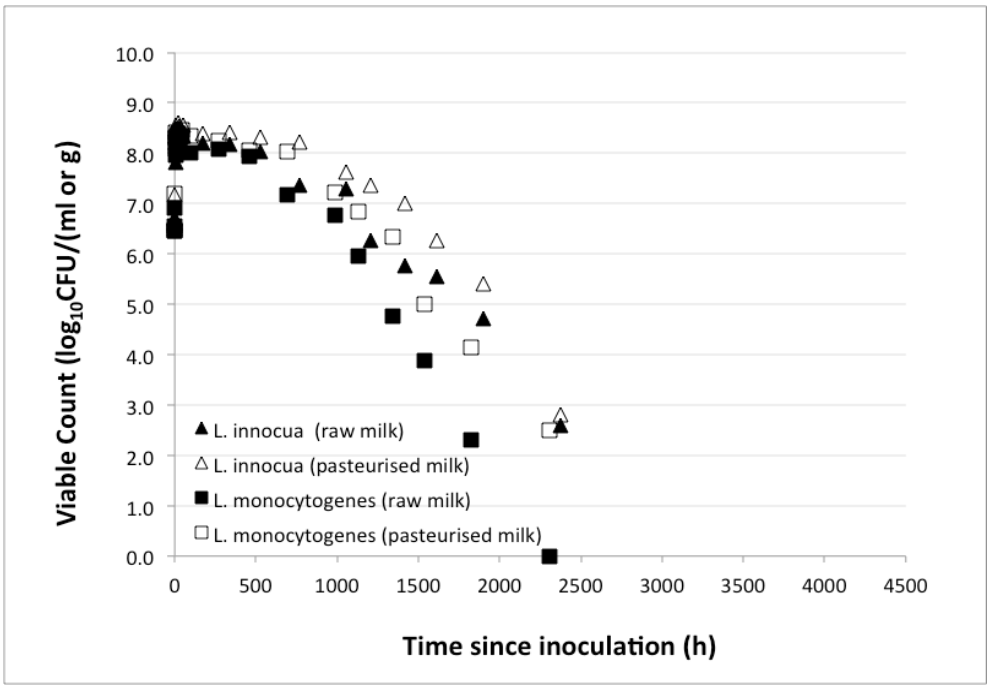


Figure 4.4a. Population changes of *L. innocua* (triangle symbols) and *L. monocytogenes* Scott A (square symbols) during the making of Feta-style cheeses made from raw milk (closed symbols) and pasteurised milk (open symbols) and matured at 15°C.

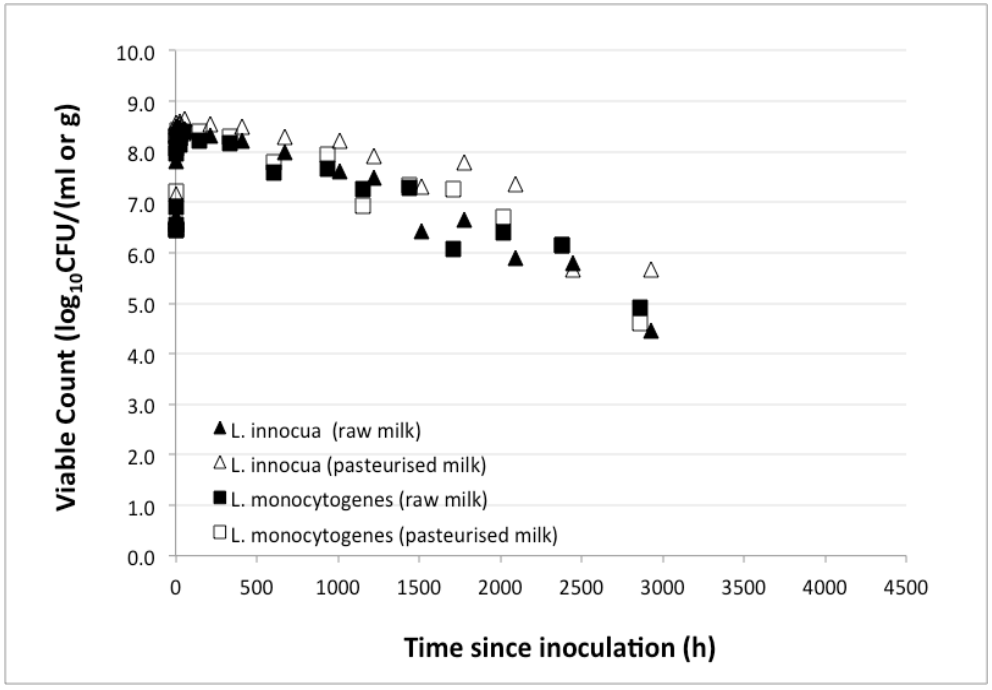


Figure 4.4b. Population changes of *L. innocua* (triangle symbols) and *L. monocytogenes* Scott A (square symbols) during the making of Feta-style cheeses made from raw milk (closed symbols) and pasteurised milk (open symbols) and matured at 5°C.

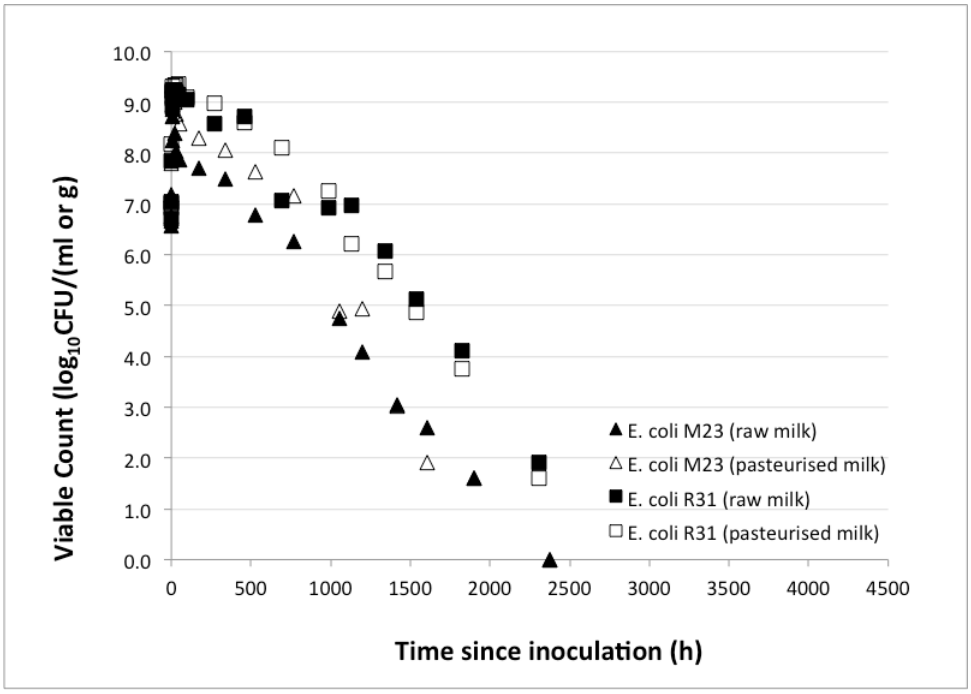


Figure 4.4c. Population changes of *E. coli* M23 (triangle symbols) and *E. coli* R31 (square symbols) during the making of Feta-style cheeses made from raw milk (closed symbols) and pasteurised milk (open symbols) and matured at 15°C.

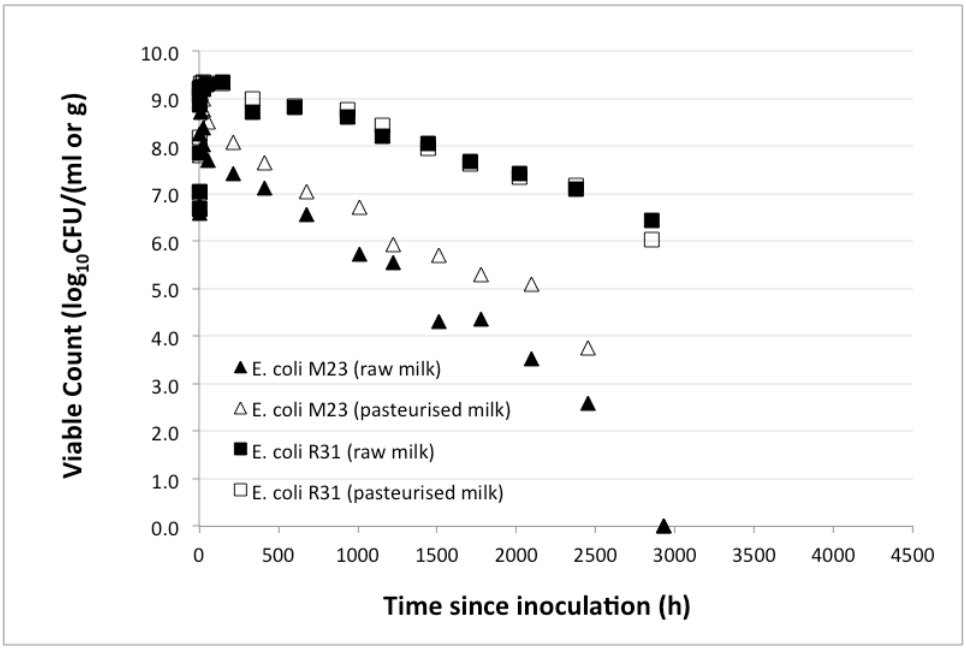


Figure 4.4d. Population changes of *E. coli* M23 (triangle symbols) and *E. coli* R31 (square symbols) during the making of Feta-style cheeses made from raw milk (closed symbols) and pasteurised milk (open symbols) and matured at 5°C.

Inactivation rates during cheese maturation were calculated by linear regression of the data (Appendix 7). The absolute rates of inactivation of the organisms were presented as Arrhenius plots including data from our earlier studies and the results of McQuestin *et al.* (2009) for *E. coli* inactivation in fermented meats for comparison (Figures 4.5a, b). This was to evaluate the potential use of existing models (*i.e.*, those generated within this project and in previous studies) to predict the inactivation rates of bacteria in actual cheeses.

Figure 4.5a. Arrhenius plot of inactivation rates for *Listeria* spp. during maturation of different cheese styles made from raw milk and pasteurised milk compared to inactivation rates for *Listeria spp.* in simulated cheese broths and those for *E. coli* in fermented meats. The red-dashed line is the model of McQuestin *et al.* (2009) for the inactivation rate of *E. coli* in fermented meats. The pale grey symbols represent the data generated in this project for *Listeria* inactivation in hard grating- and mould-ripened cheese-like broths, whereas the symbols with other colours represent the models generated for *Listeria* inactivation in the broths that emulate Brined (green), Cheddar (red) and Semi-hard (orange) cheeses. Squares and triangles indicate rates derived from challenge studies in actual cheeses; solid symbols indicate rates observed in pasteurised milk cheeses whereas open symbols indicate rates observed in raw milk cheeses.

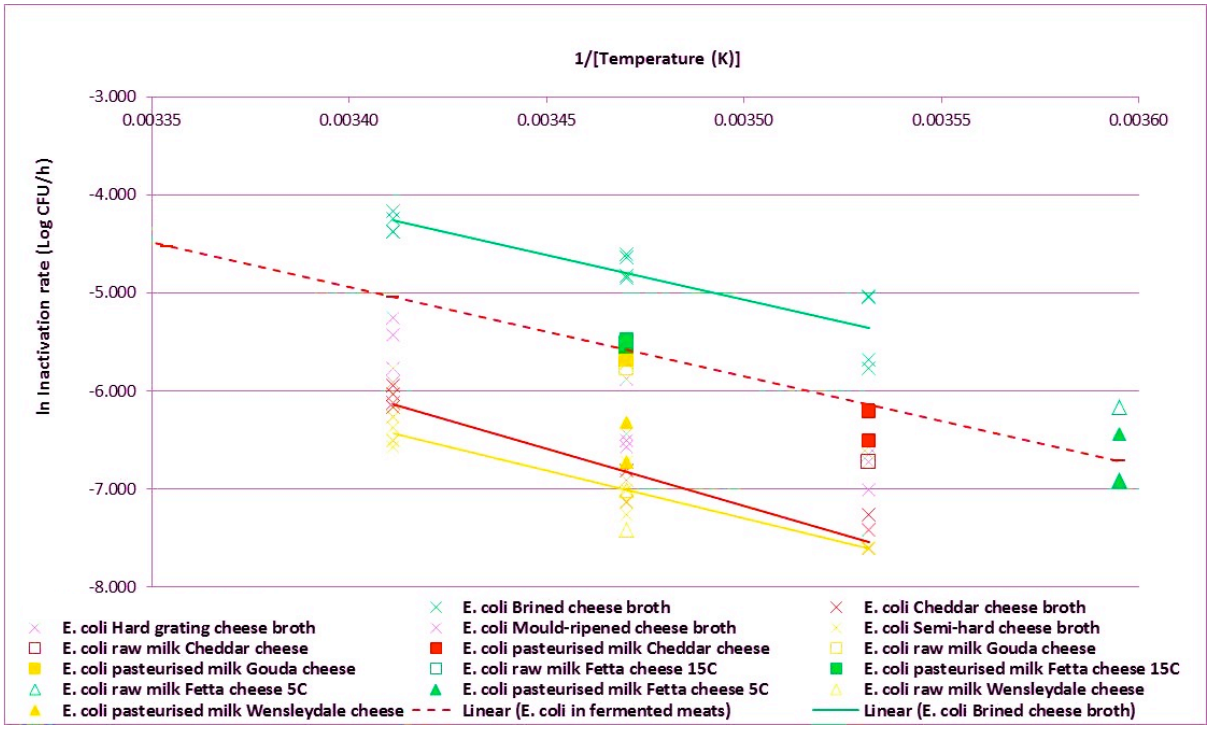


Figure 4.5b. Arrhenius plot of inactivation rates for *E. coli* during maturation of different cheese styles made from raw milk and pasteurised milk compared to inactivation rates for *E. coli* in simulated cheese broths and those for *E. coli* in fermented meats. The red-dashed line is the model of McQuestin *et al.* (2009) for the inactivation rate of *E. coli* in fermented meats. Crosses represent data generated in the present study for *E. coli* inactivation in broths with pH, water activity and lactic acid levels representative of hard-grating, mould-ripened, brined, semi-hard or cheddar cheeses. Squares and triangles indicate rates derived from challenge studies in actual cheeses; solid symbols indicated rates observed in pasteurised milk cheeses whereas open symbols indicate rates observed in raw milk cheeses.

Figures 4.5a, b show that the inactivation rates of both *Listeria* spp. and *E. coli* during maturation of all cheese types were very similar for cheese made from raw milk and the same cheese made from pasteurised milk. Figure 4.5c shows the data from Figure 4.4a superimposed on the data from Figure 4.5b to reinforce the similarity in inactivation rates between species and strains in these studies, as well as the variation in absolute inactivation rates.

This finding suggests that raw milk cheeses do not have any greater inhibitory effects on bacteria when compared to those made from pasteurised milk. This conclusion applies both to the strains and species of challenge organism used.

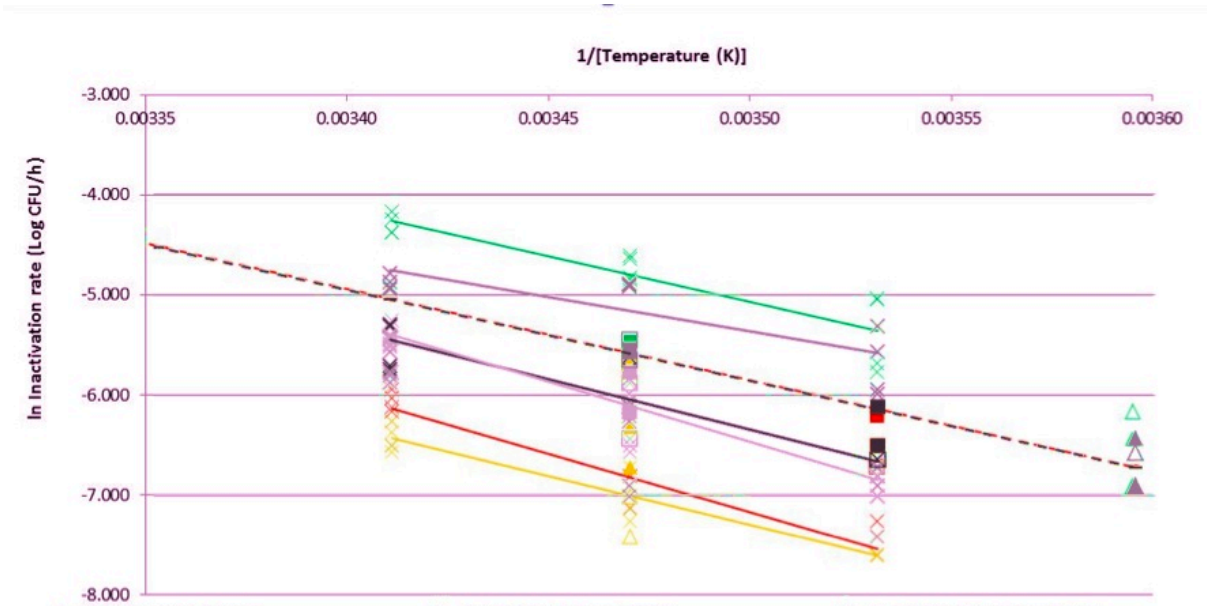


Figure 4.5c. Arrhenius plot of inactivation rates for *E. coli* (red, yellow and green symbols and lines) and *Listeria* spp. (black and purple symbols and lines) during maturation of different cheese styles made from raw milk and pasteurised milk. The figure highlights the similarity, within the range of variation, of inactivation rate responses.

It was evident that the absolute rates of inactivation of *Listeria* spp. in Cheddar and Gouda cheeses and *E. coli* in Wensleydale cheese were similar to those observed in the broths designed to emulate their respective cheese category at the same temperature tested (Figure 4.5a). By contrast, *E. coli* inactivation appeared to be faster in Cheddar and Gouda cheeses than in their analogous broths (Figure 4.5b). Inactivation of both *Listeria* spp. and *E. coli* in Feta-style cheeses was also much slower than those observed in simulated Feta cheese broths. This might be due to the differences in physico-chemical properties (e.g., pH, and lactic acid concentrations) between the specific cheeses produced and tested and the analogous broths (which were based on average or representative values for the various cheese categories). It also might be because the trials done in simulated cheese broths involved inoculation of milk-based medium designed to emulate matured cheeses (i.e. those purchased at retail stores) with challenge organisms, whereas trials involving the making of cheeses involved inoculation of milk with the challenge organisms, and production and maturation of cheeses allowing the challenge organisms time to adapt to the slowly changing conditions.

Interestingly, as evident from Figures 4.5 a and b, the inactivation rates of both *Listeria* spp. and *E. coli* in all cheese types, except for *E. coli* in Wensleydale cheese are similar to the predicted rates of *E. coli* inactivation in fermented meats at the same temperature, based on the model of McQuestin *et al.* (2009), but appear to be systematically slower for most cheese styles by a factor of 1.5 to 5. FSANZ (2014) compared the predictions of an earlier model (Ross *et al*., 2008) for the effect of temperature on inactivation rates of pathogens at non-lethal temperatures. That earlier model and data for *E. coli* inactivation rates in cheeses collated by FSANZ (2014) are compared with the model of McQuestin *et al*. (2009) and data from the current study in Figure 4.5d.

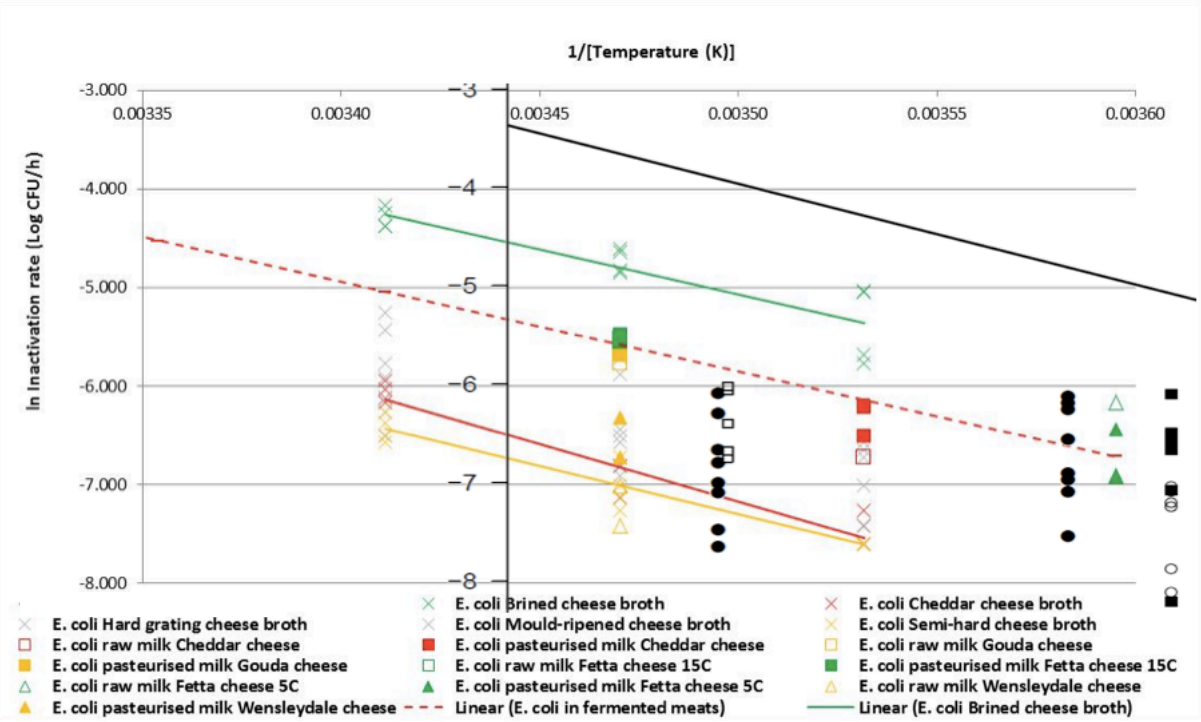


Figure 4.5d. Arrhenius plot of inactivation rates for *E. coli* during maturation of different cheese styles compared to inactivation rates for *E. coli* in simulated cheese broths and those for *E. coli* in fermented meats. The red-dashed line is the model of McQuestin *et al.* (2009) for the inactivation rate of *E. coli* in fermented meats. The pale grey symbols represent the data generated in the present study for *E. coli* inactivation in hard-grating- and mould-ripened cheese-like broths, whereas coloured symbols represent *E. coli* inactivation rates in broths that emulate cheeses. Solid symbols indicate rates derived from challenge studies in actual cheeses. The solid black line is the model of Ross *et al.* (2008) and black symbols are data for *E. coli* inactivation in cheese collated by FSANZ (2014).

That comparison further reinforces that the model of McQuestin *et al*. (2009) is more consistent with the data than the model of Ross *et al.* (2008), particularly at lower temperatures.

Changes in populations of *Listeria* spp. and *E. coli* throughout the process of making double-cream Brie-style cheese are shown in Figure 4.6a, b. Appendix 6 presents data on the chemical properties of this cheese and indicate that, as expected, as the cheese matures there is an increase in the pH of the cheese (probably due to proteolysis by enzymes from the surface moulds) that, in the absence of any other hurdle to microbial growth, allows the challenge organisms to begin to grow again. Both *Listeria* spp. and *E. coli* appeared to grow to a very high level in double-cream Brie cheese made from both raw and pasteurised milk. Also a similar pattern of responses of *Listeria* spp. and *E. coli* during production and maturation of double-cream Brie cheese was observed in cheeses made from raw and pasteurised milk. This observation is consistent with our earlier studies involving production of Wensleydale, Gouda, and Feta cheeses (*see above*).

Differences in numbers of both *E. coli* strains between double-cream Brie cheese made from raw milk and the same cheese made from pasteurised milk were observed. *E. coli* numbers appeared to be high (i.e., approximately 10^{7-8} CFU/ml) and were higher in cheese made from pasteurised milk than in raw milk cheese during the first 18 days of cheese maturation. The basis of these differences is not known but may be due to the increased growth of E. coli in pasteurised milk prior to completion of fermentation, and is considered in greater detail in Section 5. It is noted that the initial numbers of *E. coli* (i.e., approximately 10^{4-5} CFU/ml) used to inoculate milk for making double-cream Brie cheese were much lower than those (i.e., approximately 10^{6-7} CFU/ml) used to inoculate milk for making other cheese types. Higher initial numbers may alter the relationship between challenge organisms and starter cultures (the Jameson Effect) or might limit the potential for growth of the challenge organisms because the initial numbers were unrealistically high, a possibility that is also considered in greater detail in Section 5. This might explain the differences in numbers of *E. coli* between double-cream Brie cheeses made from raw or pasteurised milk, but not seen in other types of cheese.

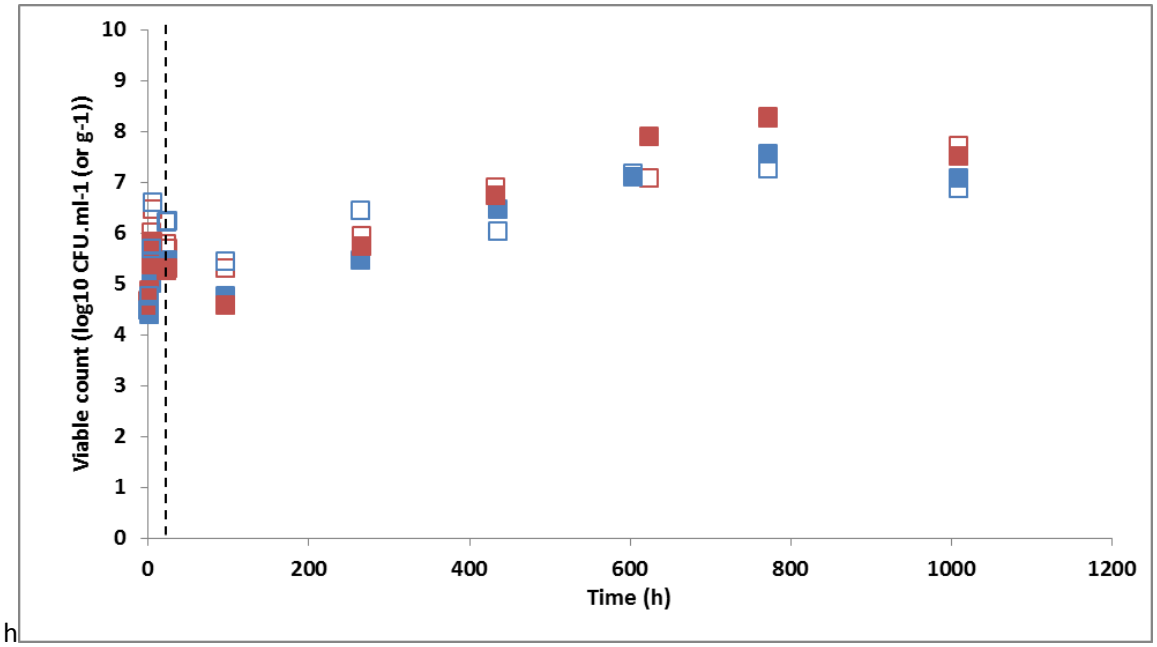
4.4 Conclusions

The data presented show that both *Listeria* spp. and *E. coli* increased in concentration during curd formation. In general, it appears that the increases were greater for *E. coli* than for *Listeria* ssp., commensurate with their respective growth rates (*see below*). A more detailed analysis is required to determine whether this is due to increased growth potential rather than concentration during curd formation. (An increase in concentration of approximately ten-fold is expected as most bacterial cells initially present in the milk are captured in the curd rather than 'lost' in the whey) and that analysis is presented in Section 5.

From initial visual assessment of the data (Figs. 4.1 - 4. 6) it appears that the increase in *E. coli* concentration during fermentation is greater than that of *Listeria* spp. Under the near 'ideal' conditions prior to commencement of fermentation (i.e., when the challenge organisms had already been added to the milk) *E. coli* growth rates would be expected to be higher (e.g., a generation time of ~25 minutes) *cf. Listeria* spp. with generation times of ~40 minutes). This trend was observed during the making of all cheese styles. However, numbers of both target organisms were unchanged for up to almost 12 days after commencement of cheese maturation.

The relevance of the increase in pathogen concentration during fermentation/curd formation/brining (i.e., when the conditions become inimical to microbial growth) is that the microbiological safety of a cheese-making process is based on:

i) initial pathogen levels
ii) increases in contamination levels due to growth or concentration prior to maturation
iii) inactivation during the maturation time.



h

Figure 4.6a. Population changes of *L. innocua* (blue) and *L. monocytogenes* Scott A (red) during the making of double-cream Brie-style cheeses made from raw milk (■ and ■) and pasteurised milk (□ and □). The dashed line differentiates the initial cheese formation (left side) from the maturation period (right side).

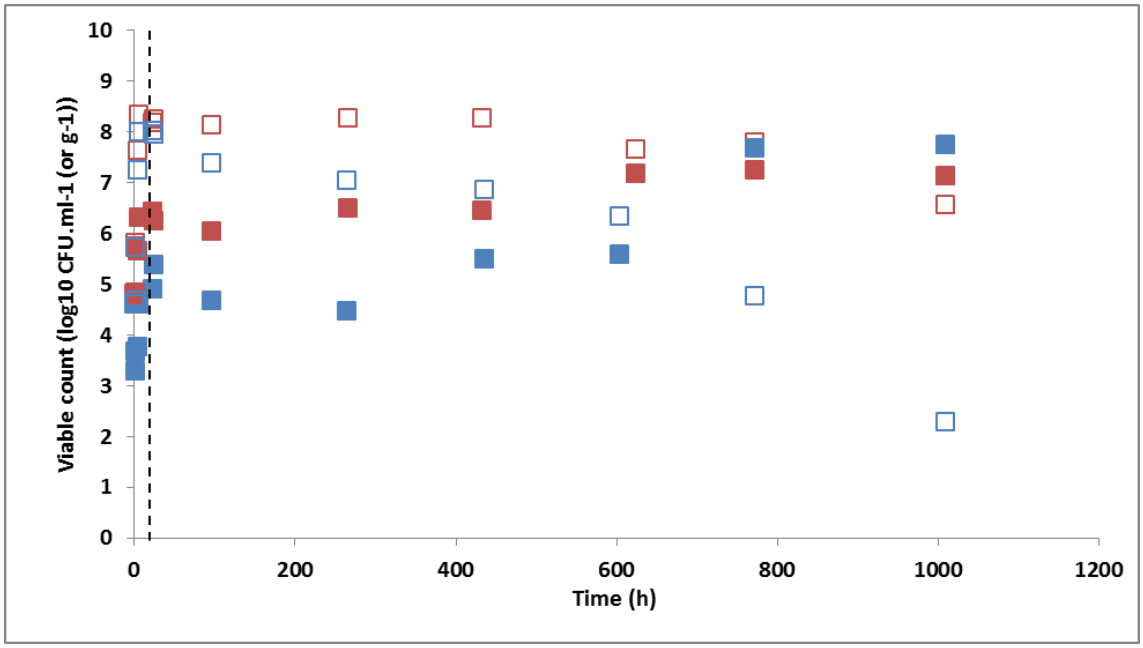


Figure 4.6b. Population changes of *E. coli* M23 (blue) and *E. coli* R31 (red) during the making of double-cream Brie-style cheeses made from raw milk (■ and ■) and pasteurised milk (□ and □). The dashed line differentiates the initial cheese formation (left side) from the maturation period (right side).

From the data presented it appears that typical inactivation levels during maturation (based on a 60 day [1440 h] maturation), are 1 – 2 logs (i.e., "factors of 10) for *Listeria* spp. and 2 – 4 logs for *E. coli*. The risk from a food depends on the concentration of pathogens present as well as the likelihood that a single cell of the pathogen could cause infection and illness. For pathogenic *E. coli*, that probability is much higher than for *L. monocytogenes* (*see* FAO/WHO, 2011, FAO/WHO, 2004) requiring that "tolerable" levels of *E. coli* in foods are much lower than "tolerable" levels of *L. monocytogenes*.

While some differences are observed in inactivation kinetics in specific cases between raw and pasteurised milk cheeses, there is no consistent difference in the inactivation kinetics of pathogens in raw milk cheese and the analogous pasteurised milk cheese. There is a suggestion, from the data, that inactivation of both pathogens is faster in pasteurised milk cheese than in raw milk cheese, but conversely that growth prior to maturation is greater for both pathogens in pasteurised milk cheese than in raw milk cheese, but the difference is very minor (~0.2 $\log_{10}$CFU difference). This is considered in greater detail in Section 5. Thus, overall, raw milk does not appear to offer any greater protection against pathogens than pasteurised milk.

The data also suggest the relevance of existing models based on *E. coli* inactivation in fermented meats to model inactivation of both organisms in cheeses, and the possibility that mechanisms of (passive) non-thermal inactivation of pathogens are not species specific. Nonetheless, the data also reveal consistent differences in inactivation rates between strains of *E. coli* and differences in inactivation kinetics of *E. coli* compared to *Listeria* spp. and also that the model, in general, predicts inactivation rates that are 2 to 5 times faster than were observed in cheeses. An additional consideration in any modelling will be to accommodate the complex inactivation kinetics in predictions of times, or conditions, to achieve a satisfactory reduction in levels of pathogens. Adopting a 'no net increase' approach, the challenge trials detailed in this Section suggest that given that pathogen loads increase due to growth and concentration from the milk into the curd, it may take from 700 to 3000 hours (1 to 4 months) before pathogen concentrations return to those initially present in the milk. If the pathogen levels are initially high, even longer times would be required to reduce pathogen loads to levels that would be considered acceptable for public health protection.

5 Pathogen responses during fermentation and curd formation

5.1 Introduction

Presented in this Section is detailed information regarding the changes in physico-chemical parameters during the processing of cheeses made from both raw and pasteurised milk, up until the commencement of the maturation stage. The data describe changes in for Cheddar, Feta, Gouda, Brie and Wensleydale-style cheeses. In addition, changes in the populations of two strains of *E. coli* (M23, and R31) and *L. monocytogenes* Scott A and the non-pathogenic *L. innocua*, inoculated into both raw and pasteurised milk that were used to make cheese, are described. The data are used to:

i) assess the influence of rate of acidification of milk during fermentation/curd formation on the potential for growth, and survival, of the introduced pathogens
ii) gauge the reproducibility of physico-chemical changes in milk/curds during fermentation (and maturation) and the fate of pathogens in particular styles of cheese
iii) assess the reliability and accuracy of predictive models for growth rate and probability of growth of *E. coli* and *Listeria* spp. in milk and cheese.

5.2 Approaches

Methods of cheese production were described briefly in Section 4.2, and are detailed in Appendix 8. Methods of analyses of pH, water activity, lactic acid and microbial concentrations are described in Appendix 1. For selected time point data, the predictions of the model of Ross *et al.* (2003) for *E. coli* growth rate and Presser *et al.* (1998) for the probability of *E. coli* growth were evaluated. Various models were consulted to ascertain representative growth rates of *L. monocytogenes* at temperatures relevant to milk acidification and curd formation and the models of Augustin *et al.* (2005) and Mejlholm and Dalgaard (2007) for *L. monocytogenes* probability of growth were also compared to observations during maturation.

Summary data for changes in challenge organism levels and pH, water activity and lactic acid levels in the cheese during ripening were essentially stable after 24 hours, with the exception of Brie-style cheeses. Also, in many of the cheeses, total lactic acid levels continued to change during ripening. Whether this was due to the reproducility/reliability of the assay could not be ascertained with confidence, but it was noted that in many cases lactic acid levels decreased somewhat during ripening. Accordingly, final pH, aw and lactic acid levels were derived as the average levels during the ripening period except for Brie-style cheeses, in which the final levels presented in the summary tables are the final values actually measured at the end of the trials.

5.3 Results and Discussion

5.3.1 Cheddar Cheese

Figures 5.1 a – h present data describing changes in physico-chemical parameters during the processing of Cheddar-style cheeses, up until the commencement of the maturation stage. Four

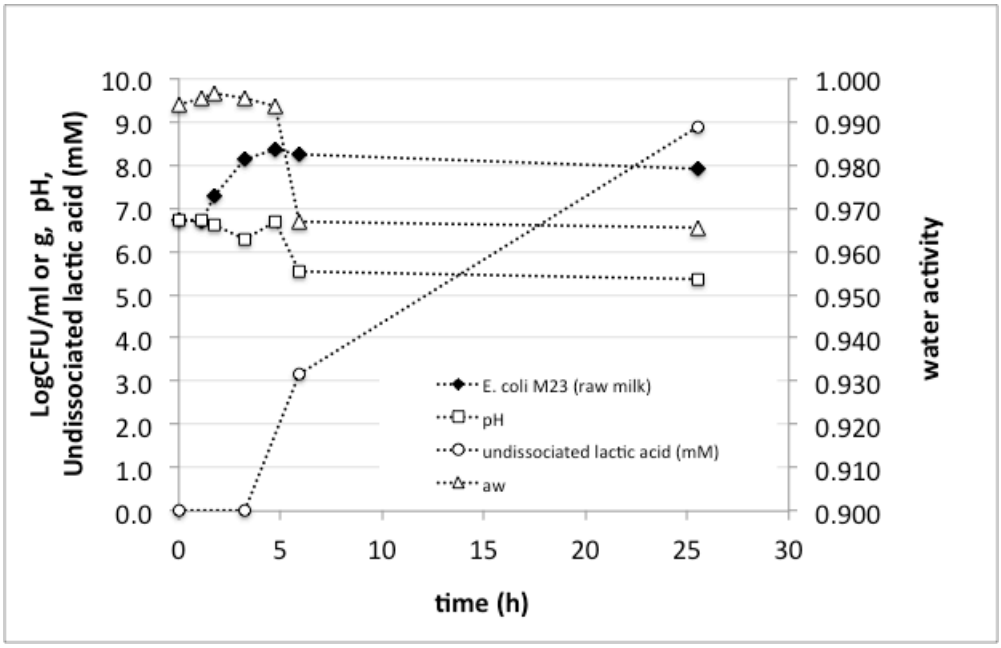
batches of Cheddar cheeses were made: two from raw milk, and two from the same milk after pasteurisation. During the processing of the cheeses one strain of *E. coli* (either R31 or M23) and one species of *Listeria*, (either *L. monocytogenes* or its non-pathogenic analogue and surrogate, *L. innocua*) were inoculated into the milk at the same time as the starter culture was added.

Table 5.1a, below, summarises the increases in levels of the introduced challenge organisms during fermentation and curd formation, changes in pH and the time that pH is stabilised after fermentation, and final water activity. The final properties are consistent with those observed in commercial cheddar style cheese (*see* Table 1). The time taken for acidification to be completed was estimated directly from the data (*see* Figures 5.1a – h) and showed that acidification was completed at approximately 5 hours after addition of cultures.

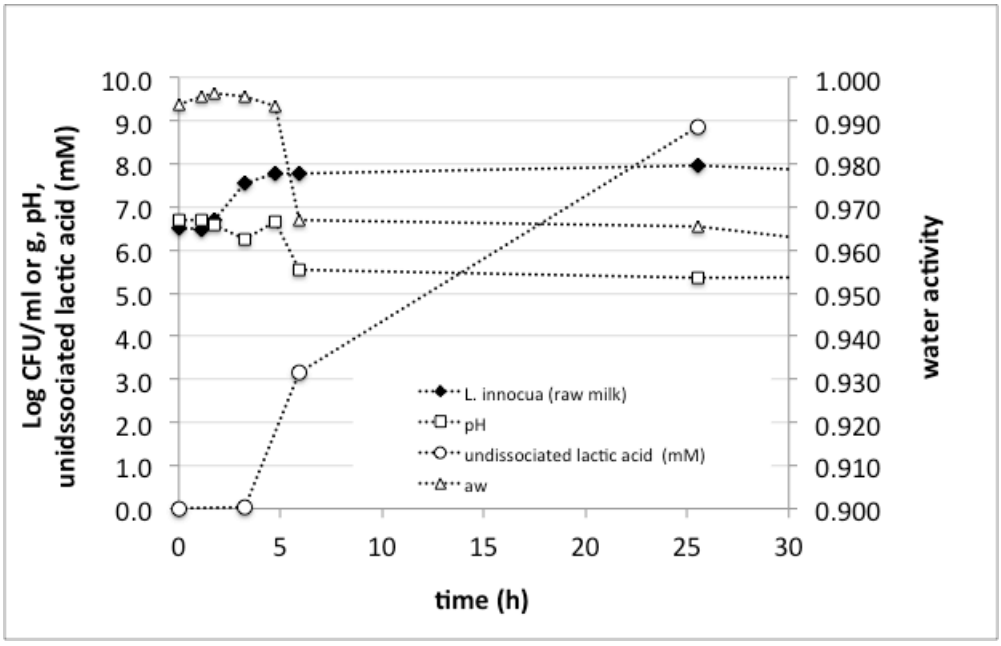
Table. 5.1a Summary data for changes in challenge organism levels and physico-chemical changes in Cheddar cheeses during fermentation/curd formation

Cheddar	*E. coli* increase (log_{10}CFU)		*Listeria* ssp. increase (log_{10}CFU)		pH change	Time taken (h)	"Final" pH	"Final" a_w
Batch	M23	R31	*mono-cytogenes*	*innocua*				
Raw 1	1.65			1.45	1.4	~5	5.36	0.949
Raw 2		2.00	1.06		1.4	~5	5.33	0.953
Pasteurised 1	1.85			1.65	1.4	~5	5.25	0.953
Pasteurised 2		2.12	1.17		1.6	~5	5.26	0.940
Log difference pasteurised to raw	0.20	0.12	0.11	0.20			Final lactic acid (%w/w)	0.61 – 0.95
Average increase *Listeria*	**1.33**	*0.31*	(SD)					
Average increase *E .coli*	**1.91**	*0.18*	(SD)					
Incubation to curd processing:	1.5 h x 32°C, then to 38°C over 30 min. and held at 38°C until salt added							
Starter culture:	type A							

a)

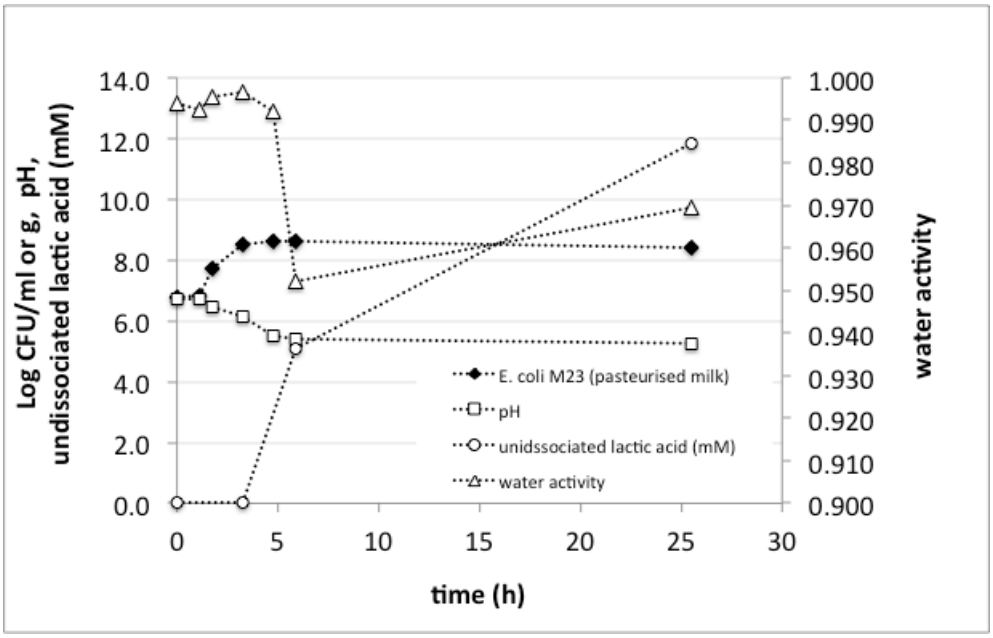


b)



Figures 5.1a, b. Changes in pH, water activity and undissociated lactic acid concentration in Cheddar cheese made from raw milk. Figure 5.1 a (upper) also shows changes in levels of *E. coli* M23 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.1b (lower) shows changes in levels of *L. innocua* inoculated into the milk at the same time as addition of starter cultures.

c)



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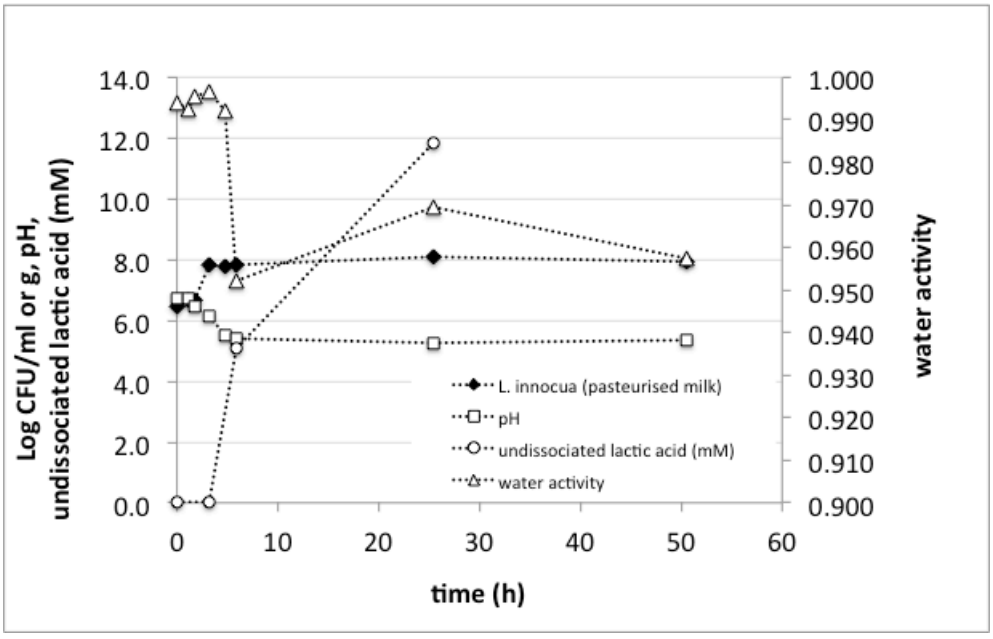
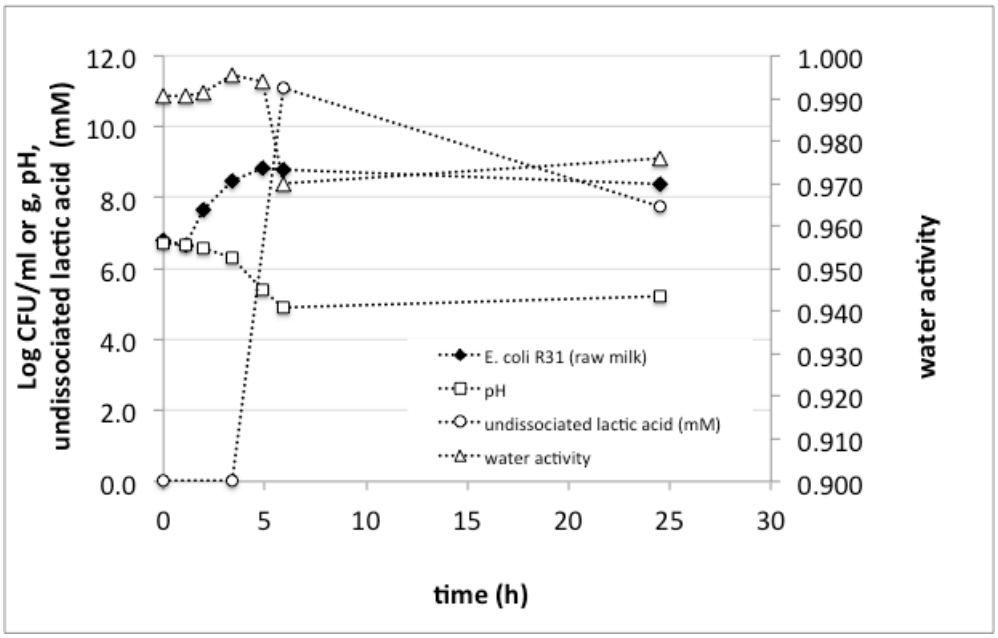
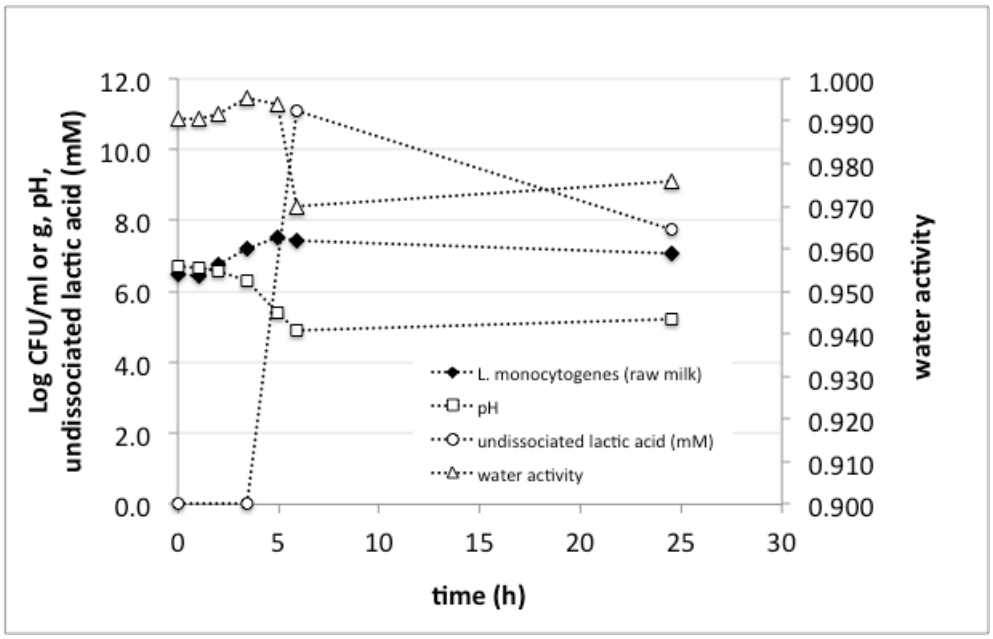


Figures 5.1c, d. Changes in pH, water activity and undissociated lactic acid concentration in Cheddar cheese made from pasteurised milk. Figure 5.1 c (upper) also shows changes in levels of *E. coli* M23 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.1d (lower) shows changes in levels of *L. innocua* inoculated into the milk at the same time as addition of starter cultures.

e)

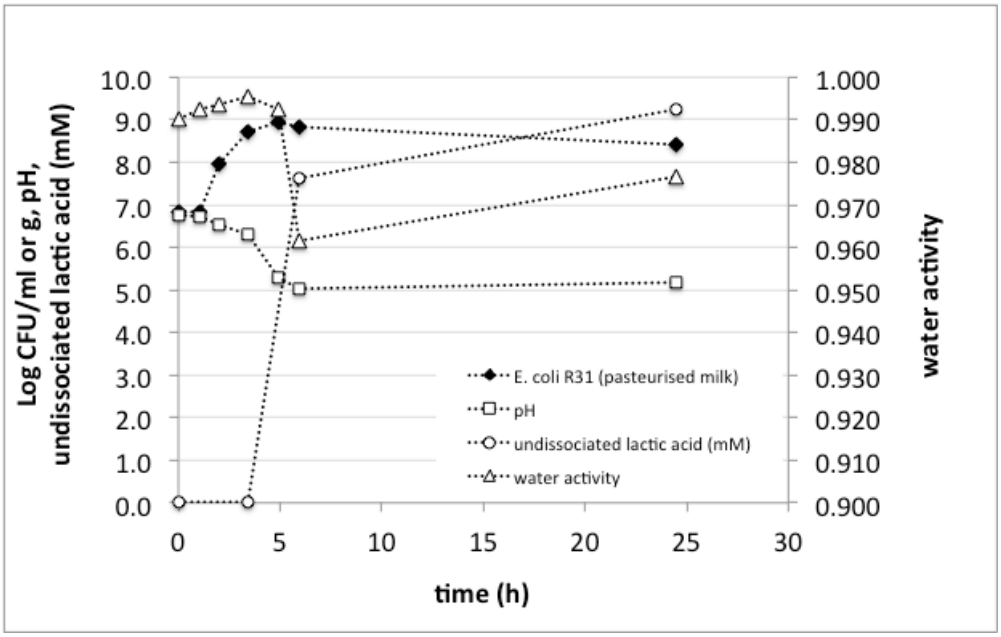


f)

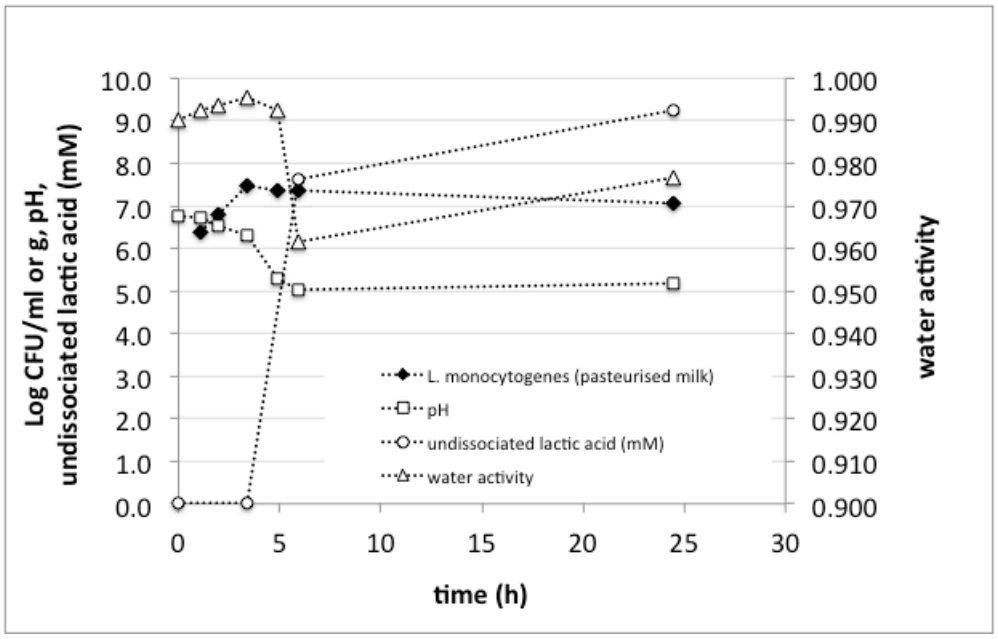


Figures 5.1e, f. Changes in pH, water activity and undissociated lactic acid concentration in Cheddar cheese made from raw milk. Figure 5.1e (upper) also shows changes in levels of *E. coli* R31 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.1f (lower) show**s c**hanges in levels of *L. monocytogenes* Scott A inoculated into the milk at the same time as addition of starter cultures.

g)



h)



Figures 5.1g, h. Changes in pH, water activity and undissociated lactic acid concentration in Cheddar cheese made from pasteurised milk. Figure 5.1g (upper) also shows changes in levels of *E. coli* R31 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.1h (lower) show**s c**hanges in levels of *L. monocytogenes* Scott A inoculated into the milk at the same time as addition of starter cultures.

Increases in *E. coli* levels were greater than increases in levels of *Listeria* strains, and the final pH and water activity were very consistent between all batches made, whether from raw or pasteurised milk and independent of the challenge organisms introduced. This degree of consistency between independent batches of the same style of cheese differs from that observed in batches of commercial cheeses from the same producers but at different times, as described in Section 1. Also noteworthy is that the *increases* in challenge organism levels were slightly higher in all cheeses made from pasteurised milk compared to the same cheeses made from raw milk.

The increase in levels is expected and is, at least in part, a consequence of the concentration of the cells into the curd, expected to lead to an approximately ten-fold ("one log") increase. Generation times of *L. monocytogenes*, at pH 6.5, and derived from the models of Devlieghere *et al*. (2001), Grau and Vanderlinde (1993) and ComBase (www.cc.combase) are expected to be approximately 35 – 40 minutes throughout the range from 32 to 38°C. For *Escherichia coli*, generation times derived from the model of Ross *et al.* (2003) the ComBase are ~24 minutes at 32°C and ~20 minutes at 34-38°C.

From the data, fastest growth rates in each trial for each challenge organism were estimated by linear regression of data (judged from the plots) considered to represent the most rapid increase in $\log_{10}$ bacterial numbers. These data usually comprised two or three time points and corresponding $\log_{10}$CFU values. The estimates are presented in Table 5.1b, below. In general the observed growth rates are in good agreement with model predictions. However, the *extent* of growth is less than would be expected in the four hours of curd formation if growth were not inhibited by other factors. From Figures 5.1 it can be seen that growth is only evident for approximately 3 hours before the population growth rate declines to zero. While the growth rate of all challenge organisms declined to zero after addition of salt, growth rate had begun to decline as pH fell/lactic acid concentration increased. It is also possible that, due to the relatively high starting inoculum, the challenge organisms reached stationary phase (i.e., achieved their maximum cell density) and that this also contributed to the observed growth rate decline. This possibility will be discussed further later.

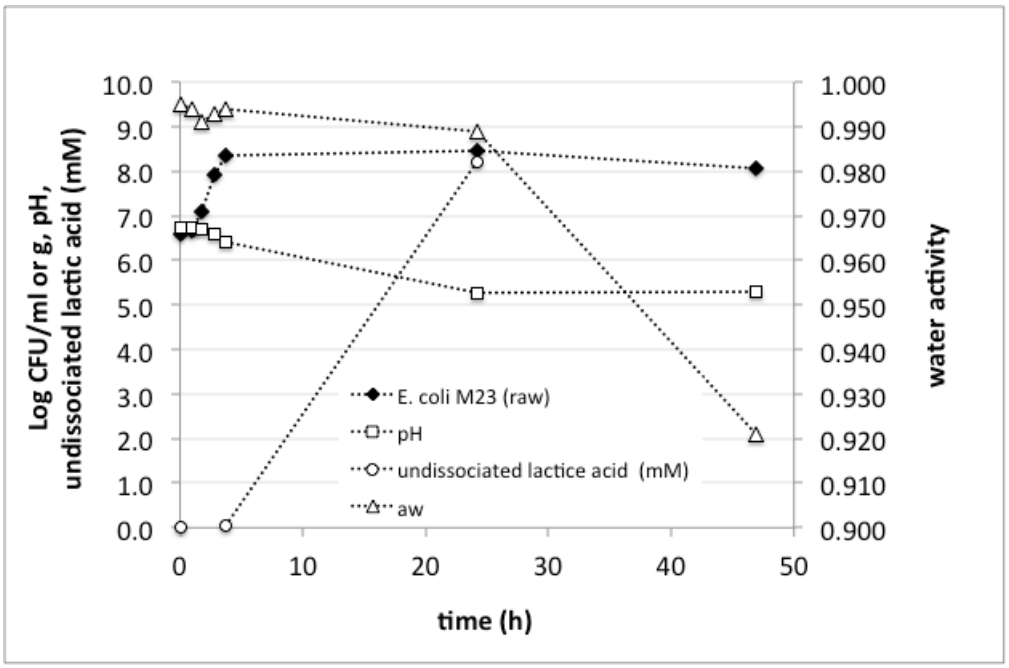
Table. 5.1b Generation time estimates for challenge organism in Cheddar cheeses during fermentation/curd formation

Cheddar	Estimated *E. coli* gen time (min)		Estimated *Listeria* gen time (min)	
Batch	M23	R31	*L. monocytogenes*	*L. innocua*
Raw 1	28			36
Raw 2		24	64	
Pasteurised 1	25			24
Pasteurised 2		23	40	

5.3.2 Gouda Cheese

Figures 5.2a – h present data describing changes in physico-chemical parameters during the processing of Gouda-style cheeses, up until the commencement of the maturation stage. Four batches of cheddar cheeses were made: two from raw milk, and two from the same milk after pasteurisation. During the processing of the cheeses one strain of *E. coli* (either R31 or M23) and one species of *Listeria*, (either *L. monocytogenes* or its non-pathogenic analogue and surrogate, *L. innocua*) were inoculated into the milk at the same time as the starter culture was added.

a)



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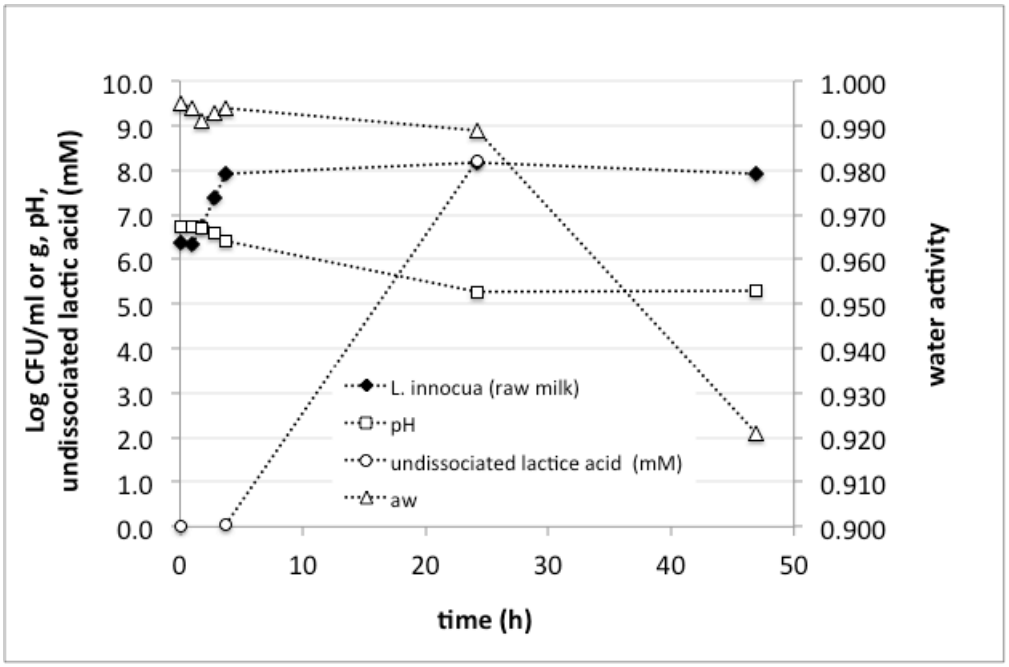
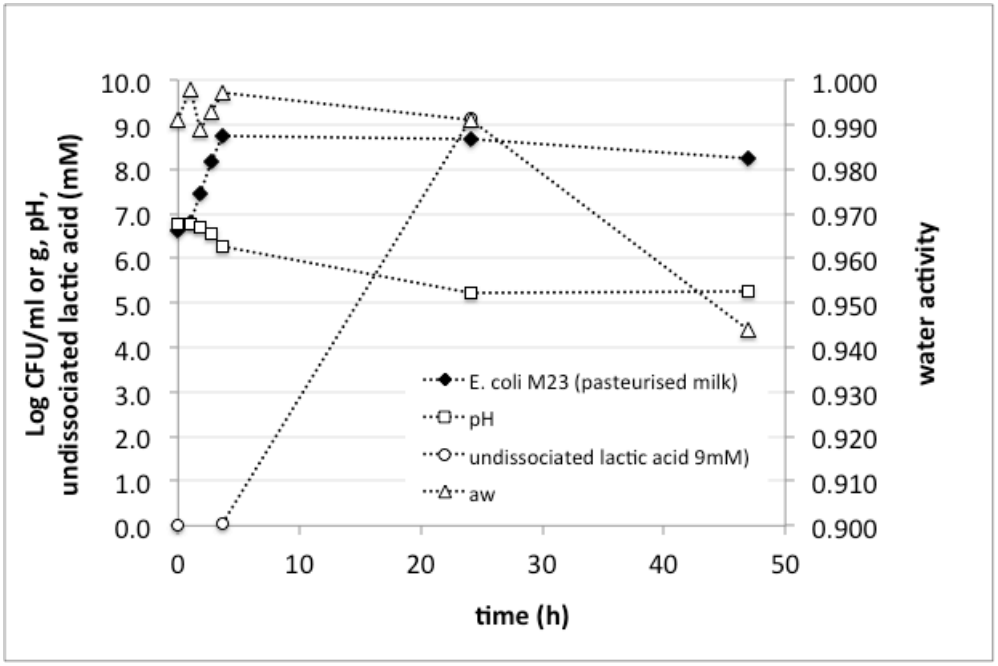


Figures 5.2a, b. Changes in pH, water activity and undissociated lactic acid concentration in Gouda cheese made from raw milk. Figure 5.2a (upper) also shows changes in levels of *E. coli* M23 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.2b (lower) shows changes in levels of *L. innocua* inoculated into the milk at the same time as addition of starter cultures.

c)



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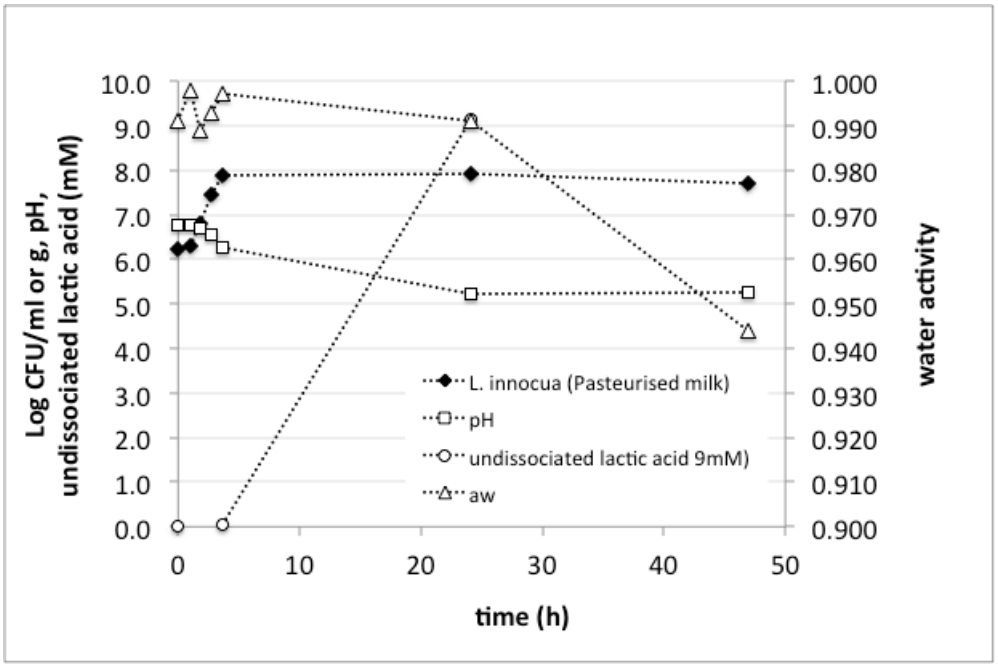
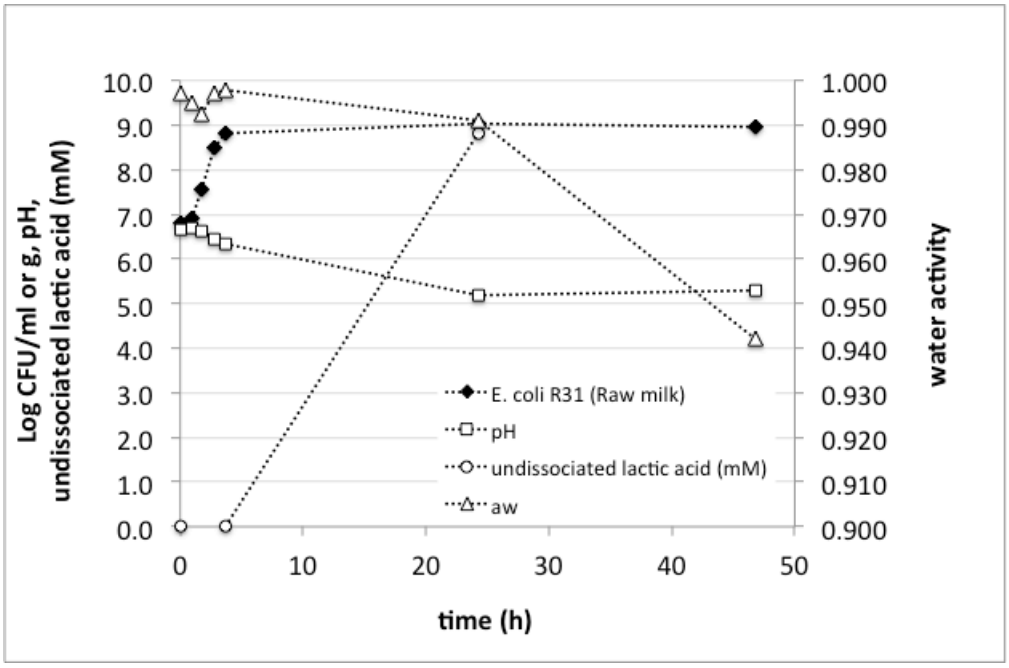


Figures 5.2c, d. Changes in pH, water activity and undissociated lactic acid concentration in Gouda cheese made from pasteurised milk. Figure 5.2c (upper) also shows changes in levels of *E. coli* M23 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.2d (lower) show**s c**hanges in levels of *L. innocua* inoculated into the milk at the same time as addition of starter cultures.

e)



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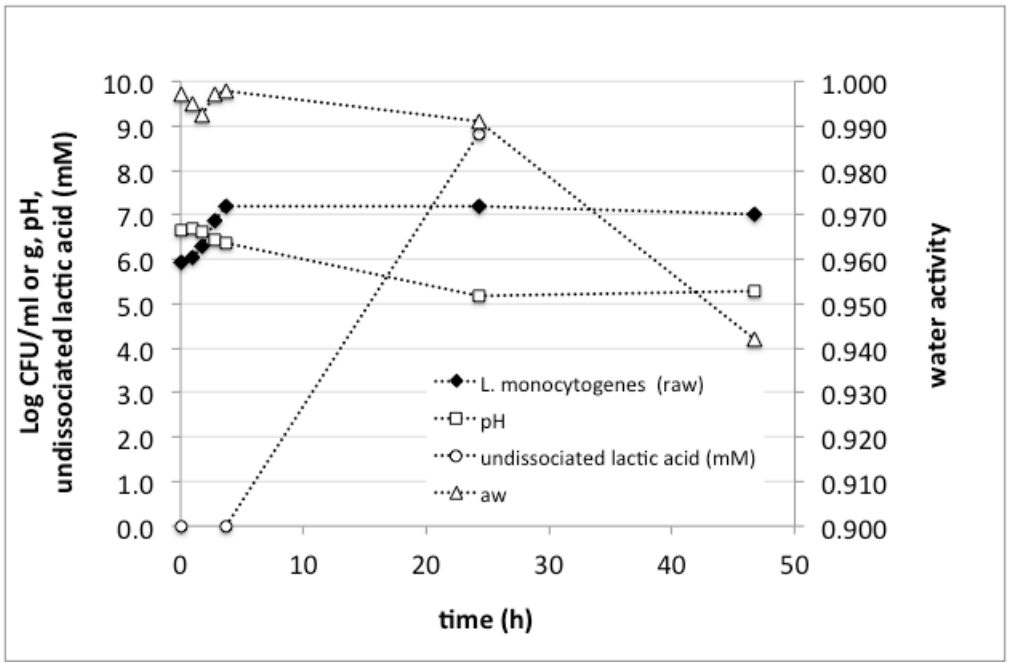
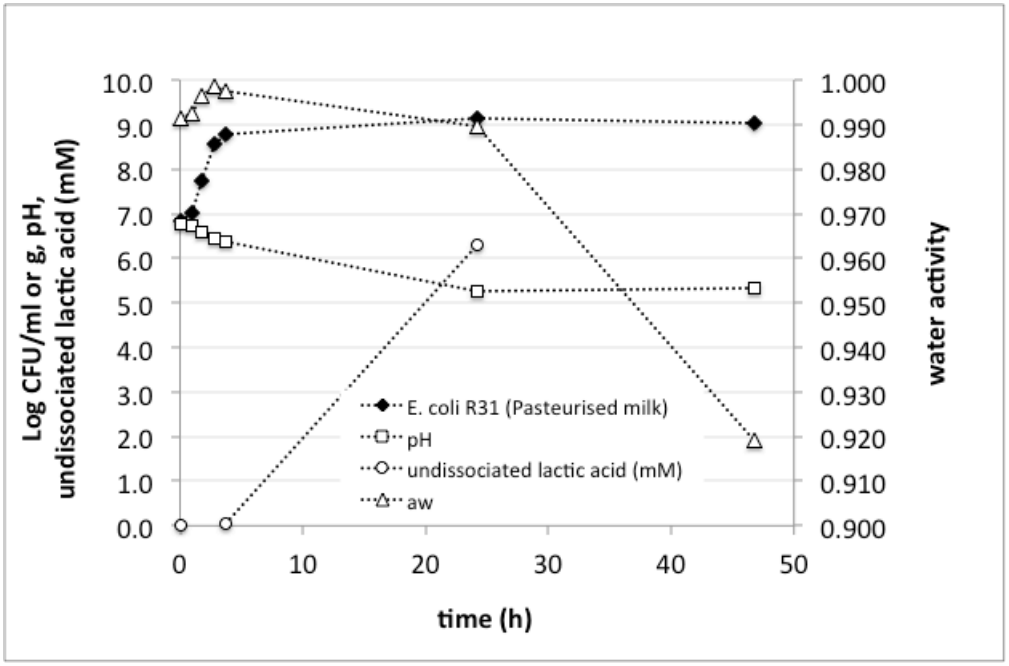


Figs. 5.2e, f. Changes in pH, water activity and undissociated lactic acid concentration in Gouda cheese made from raw milk. Figure 5.2e (upper) also shows changes in levels of *E. coli* R31 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.2f (lower) show**s c**hanges in levels of *L. monocytogenes* Scott A inoculated into the milk at the same time as addition of starter cultures.

g)



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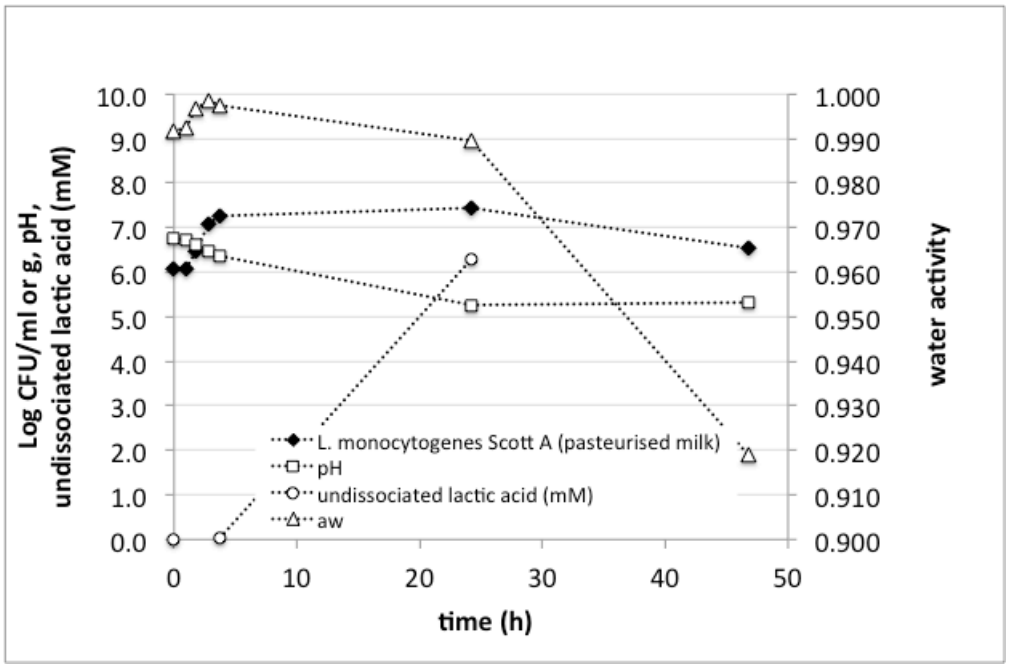


Figures 5.2g, h. Changes in pH, water activity and undissociated lactic acid concentration in Gouda cheese made from pasteurised milk. Figure 5.2g (upper) also shows changes in levels of *E. coli* R31 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.2h (lower) shows changes in levels of *L. monocytogenes* Scott A inoculated into the milk at the same time as addition of starter cultures.

Table 5.2a presents summary data for the changes in levels of challenge organisms and physico-chemical parameters of Gouda cheeses during curd formation and processing. Table 5.2b presents generation time estimates for each culture in each Gouda cheese that was produced, using the same methods as described in Section 5.3.1. The time taken for acidification to be completed was estimated from the data (*see* Figures 5.2a – h) by approximation by extrapolating the observed rate of acidification to the final pH achieved.

Table. 5.2a Summary data for changes in challenge organism levels and physico-chemical changes in Gouda cheeses during fermentation/curd formation

Gouda	*E. coli* increase (log_{10}CFU)		*Listeria* ssp. increase (log_{10}CFU)		pH change	Time taken (h)	"Final" pH	"Final" a_w
Batch	M23	R31	*mono-cytogenes*	*innocua*				
Raw 1	1.89			1.77	1.4	~ 10	5.4	0.935
Raw 2		2.24	1.29		1.4	~ 10	5.36	0.932
Pasteurised 1	2.11			1.69	1.5	~ 10	5.4	0.938
Pasteurised 2		2.32	1.35		1.5	~ 10	5.37	0.933
Log difference pasteurised to raw	0.22	0.08	0.06	0.08			Final lactic acid (%w/w)	0.65 – 0.74
Average increase *Listeria*	**1.53**	*0.22*	(SD)					
Average increase *E .coli*	**2.14**	*0.18*	(SD)					
Incubation to curd processing:	2.5 h x 32°C then raised to 38°C							
Starter culture:	type B							

As observed in Cheddar-style cheeses (Section 5.3.1), increases in *E. coli* levels were greater than increases in levels of *Listeria* spp., and the final pH and water activity were very consistent between all batches made, whether from raw or pasteurised milk and independent of the challenge organisms introduced. Also as observed in Cheddar-style cheeses, the increases in challenge organism levels were higher in all cheeses made from pasteurised milk compared to the same cheeses made from raw milk. Generation time estimates for each strain/species of challenge organism (Table 5.2b) are similar to those observed during processing of Cheddar cheeses. Also, as observed in the Cheddar cheese challenge trials, growth inhibition seemed to correspond to declining pH and increasing lactic acid levels, rather than a_w changes.

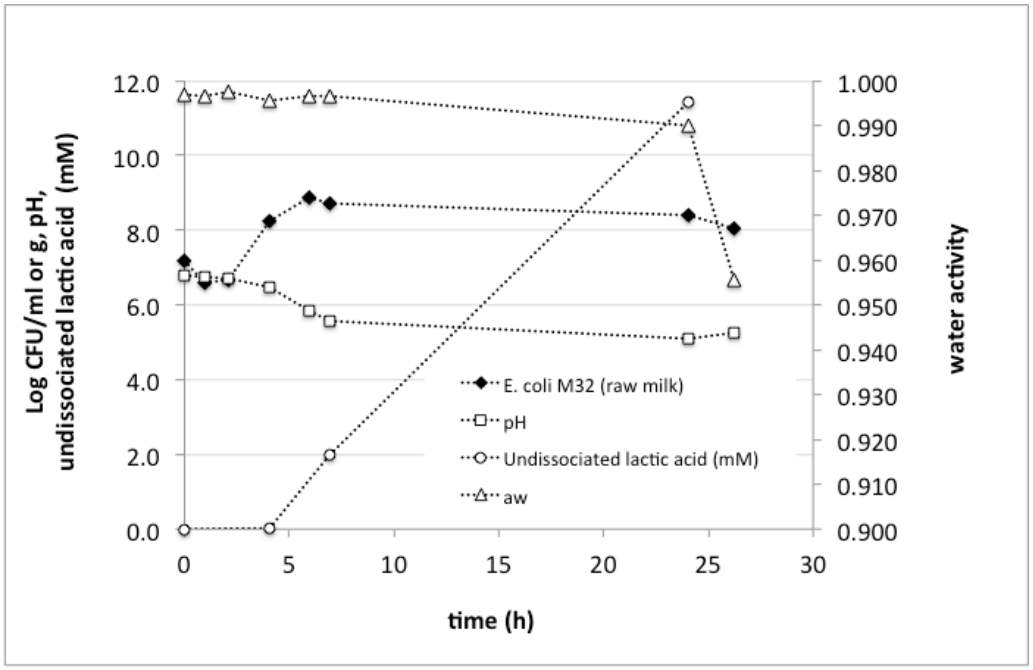
Table. 5.2b Generation time estimates for challenge organisms in Gouda cheeses during fermentation/curd formation

Gouda	Estimated *E. coli* gen time (min)		Estimated *Listeria* gen time (min)	
Batch	M23	R31	*L. monocytogenes*	*L. innocua*
Raw 1	25			31
Raw 2		26	40	
Pasteurised 1	25			28
Pasteurised 2		28	33	

5.3.3 Feta Cheese

Figures 5.3a – h present data describing changes in physico-chemical parameters during the processing of Feta-style cheeses, up until the commencement of the maturation stage. The data are summarised in Table 5.3a. Four batches of Feta cheeses were made: two from raw milk, and two from the same milk after pasteurisation. During the processing of the cheeses one strain of *E. coli* (either R31 or M23) and one species of *Listeria*, (either *L. monocytogenes* or its non-pathogenic analogue and surrogate, *L. innocua*) were inoculated into the milk at the same time as the starter culture was added. Each batch of cheeses (raw or pasteurised milk, and different combinations of challenge organisms) was subsequently split into equal portions and one set of batches matured at 15°C, and a second set of Feta-style cheese matured at 5°C. The data presented relate to the physico-chemical parameters, and microbiological changes, in each batch prior to them being separated and stored at different temperatures. For each strain/species of challenge organism in each batch produced, generation times were estimated from the data as described in Section 5.3.1 and the estimates presented in Table 5.3b.

a)



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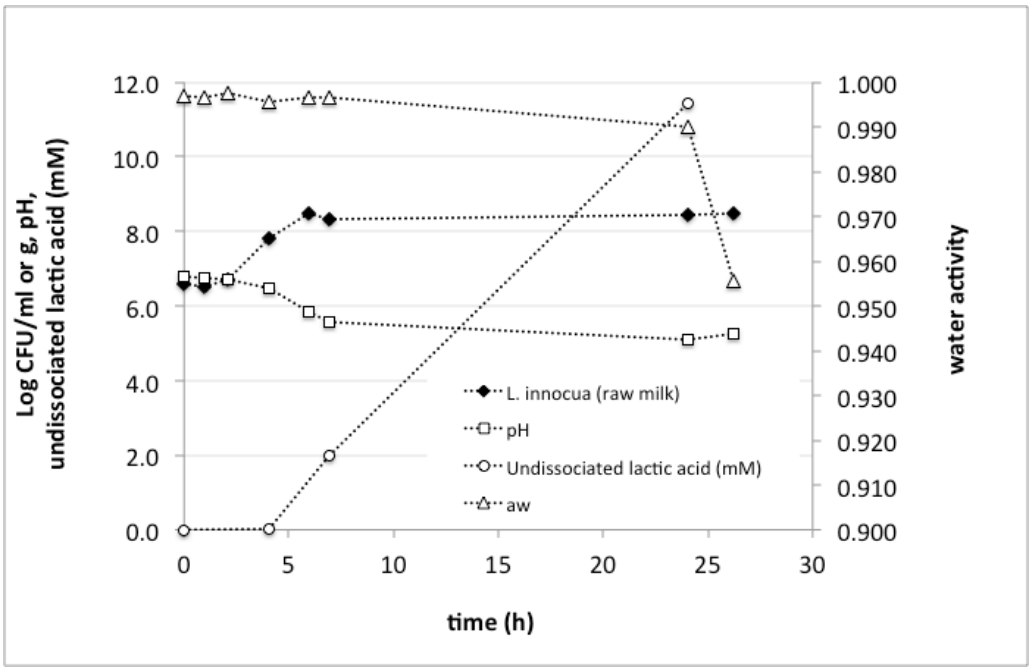
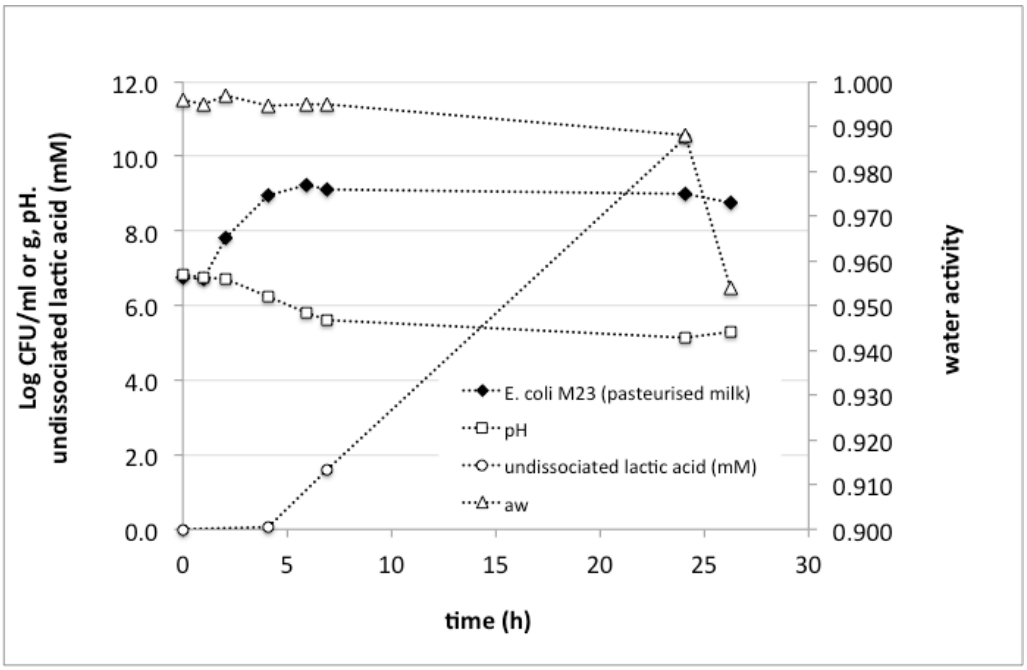


Figures 5.3a, b. Changes in pH, water activity and undissociated lactic acid concentration in Feta cheese made from raw milk. Figure 5.3a (upper) also shows changes in levels of *E. coli* M23 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.3b (lower) show**s c**hanges in levels of *L. innocua* inoculated into the milk at the same time as addition of starter cultures.

c)



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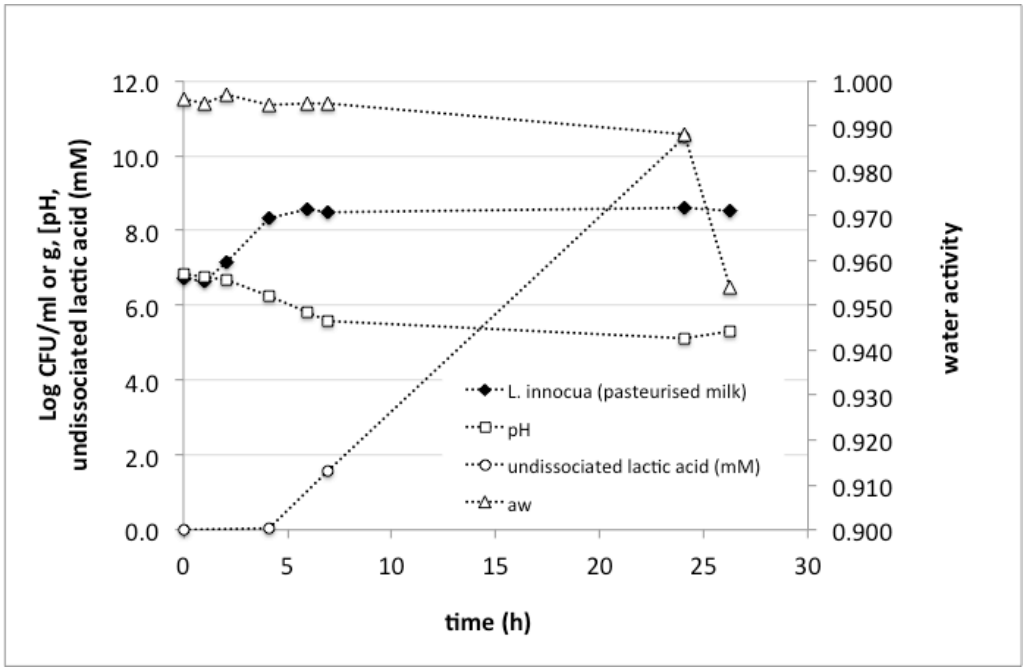
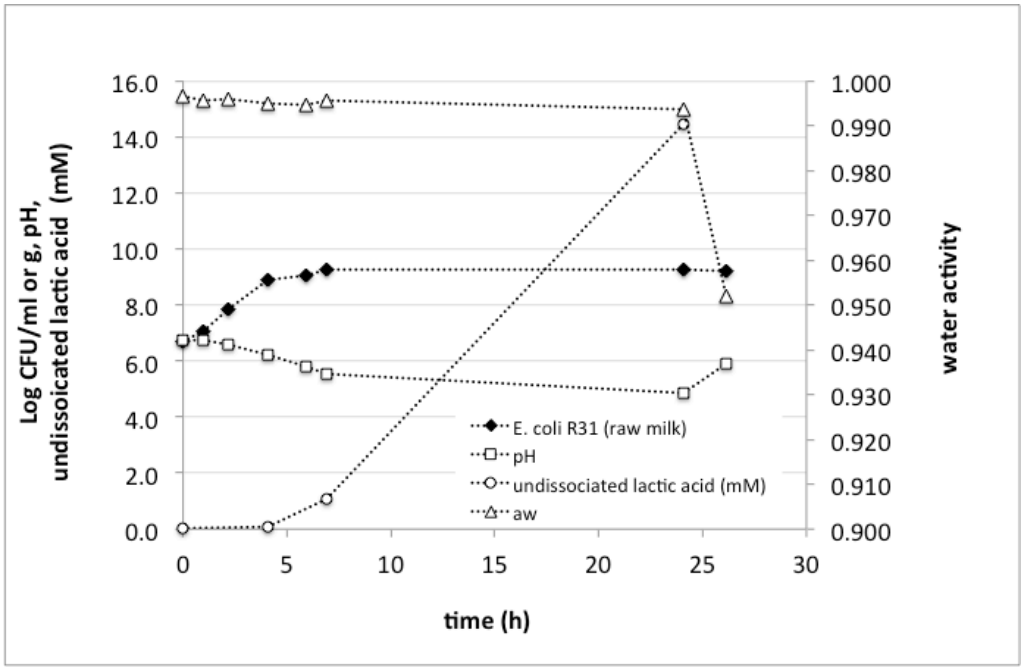


Figure 5.3c, d. Changes in pH, water activity and undissociated lactic acid concentration in Feta cheese made from pasteurised milk. Figure 5.3c (upper) also shows changes in levels of *E. coli* M23 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.3d (lower) shows changes in levels of *L. innocua* inoculated into the milk at the same time as addition of starter cultures.

e)



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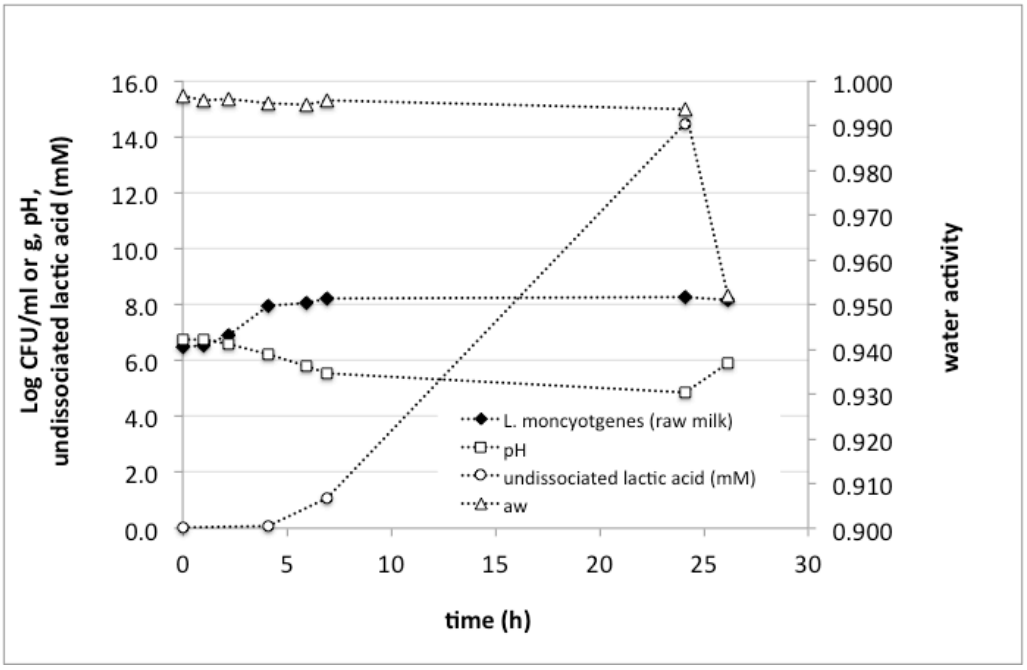
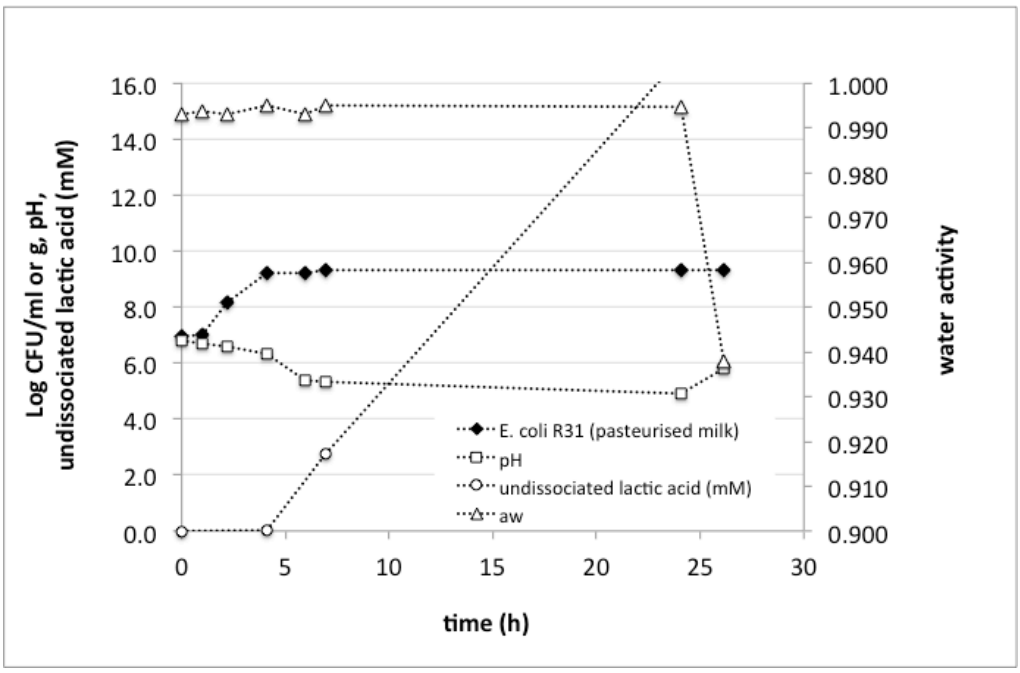


Figures 5.3e, f. Changes in pH, water activity and undissociated lactic acid concentration in Feta cheese made from raw milk. Figure 5.3e (upper) also shows changes in levels of *E. coli* R31 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.3f (lower) show**s c**hanges in levels of *L. monocytogenes* Scott A inoculated into the milk at the same time as addition of starter cultures.

g)



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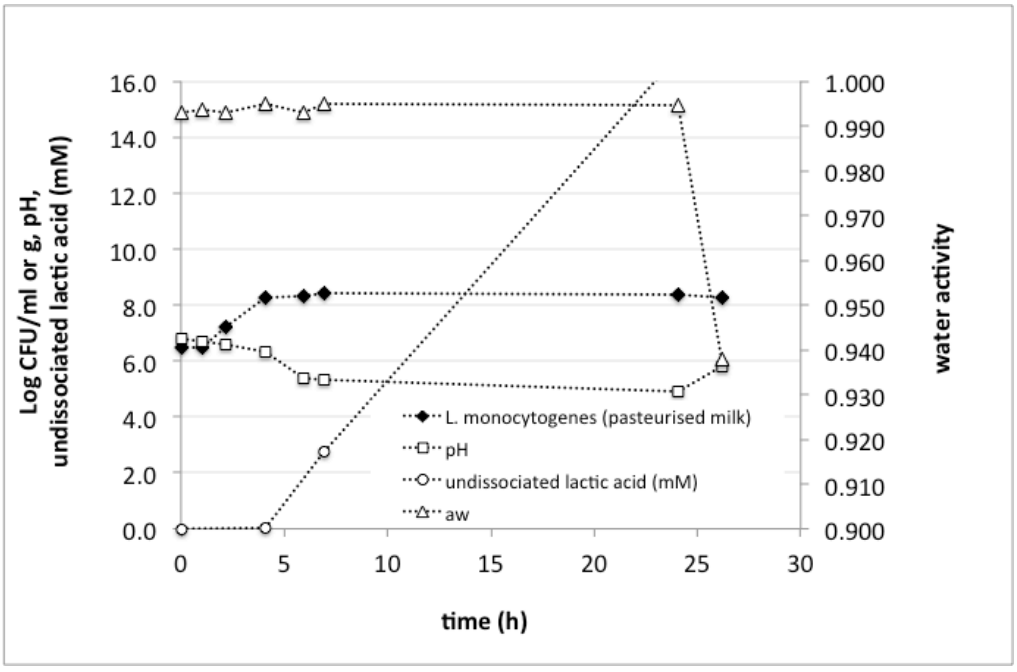


Figure 5.3g, h. Changes in pH, water activity and undissociated lactic acid concentration in Feta cheese made from pasteurised milk. Figure 5.3g (upper) also shows changes in levels of *E. coli* R31 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.3h (lower) shows changes in levels of *L. monocytogenes* Scott A inoculated into the milk at the same time as addition of starter cultures.

Table 5.3a presents summary data for the changes in levels of challenge organisms and physico-chemical parameters of Feta cheeses during curd formation and processing. Table 5.3b presents generation time estimates for each culture in each Feta cheese that was produced, using the same methods as described in Section 5.3.1. The time taken for acidification to be completed was estimated directly from the plotted data (*see* Figures 5.3a – h). While the water activity is typical of brined cheeses (see Table 1) the pH is somewhat higher than expected for a brined cheese.

Table. 5.3a Summary data for changes in challenge organism levels and physico-chemical changes in Feta cheeses during fermentation/curd formation

Feta	*E. coli* increase (log_{10}CFU)		*Listeria* ssp. increase (log_{10}CFU)		pH change	Time taken (h)	"Final" pH	"Final" a_w
Batch	M23	R31	*mono-cytogenes*	*innocua*				
Raw 1	2.28			1.89	1.68	~ 8	5.11	0.953
Raw 2		2.55	1.89		1.59	~ 8	5.16	0.954
Pasteurised 1	2.48			1.88	1.64	~ 8	5.18	0.954
Pasteurised 2		2.43	1.97		1.6	~ 8	5.19	0.947
Log difference pasteurised to raw	0.20	-0.12	0.08	-0.01			Final lactic acid (%w/w)	0.63 – 0.84
Average increase *Listeria*	**1.91**	*0.05*	(SD)					
Average increase *E .coli*	**2.44**	*0.14*	(SD)					
Incubation to curd processing:	2 to 2.5 h x 34°C							
Starter culture:	type A							

Table. 5.3b Generation time estimates for challenge organisms in Feta cheeses during fermentation/curd formation

Feta	Estimated *E. coli* gen time (min)		Estimated *Listeria* gen time (min)	
Batch	M23	R31	*L. monocytogenes*	*L. innocua*
Raw 1	23			39
Raw 2		30	33	
Pasteurised 1	26			33
Pasteurised 2		18	31	

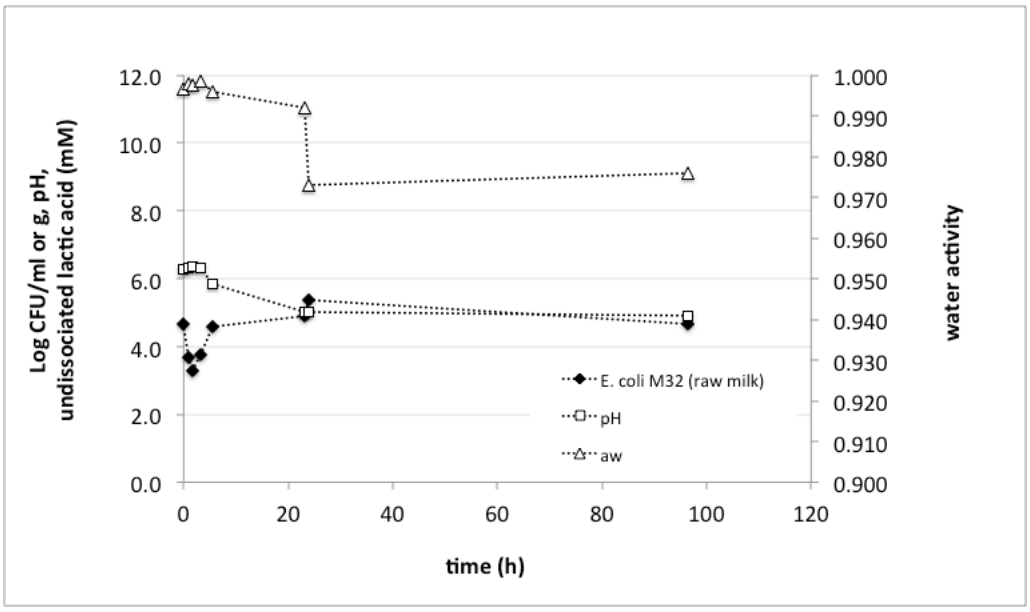
As observed in Cheddar-style cheeses (Section 5.3.1) and Gouda-style cheeses (Section 5.3.2), increases in *E. coli* levels were greater than increases in levels of *Listeria* spp., and the final pH and water activity were very consistent between all batches made, whether from raw or pasteurised milk and independent of the challenge organisms introduced. In contrast to Cheddar-style and Gouda-style cheeses, however, the increases in challenge organism levels were not consistently higher in cheeses made from pasteurised milk compared to the same cheeses made from raw milk. Generation time estimates for each strain/species of challenge organism (Table 5.2b) are similar to those observed during processing of Cheddar and Gouda cheeses. On average, the generation times estimated from the data are consistent with predicted growth rates in milk at 32 – 38°C, with slower growth of *Listeria* spp. than *E. coli* consistently observed.

5.3.4 Double-cream Brie Cheese

Figures 5.4 a – h present data describing changes in physico-chemical parameters during the processing of double-cream Brie-style cheeses, up until the commencement of the maturation stage. Four batches of cheddar cheeses were made: two from raw milk, and two from the same milk after pasteurisation. During the processing of the cheeses one strain of *E. coli* (either R31 or M23) and one species of *Listeria*, (either *L. monocytogenes* or its non-pathogenic analogue and surrogate, *L. innocua*) were inoculated into the milk at the same time as the starter culture was added. The changes in physico-chemical properties of the developing cheese, and changes in challenge organism levels are summarised in Table 5.4a, together with pH and water activity levels at the commencement of maturation.

Generation times for each organism in each cheese were estimated by linear regression of appropriate data, as described in Section 5.3.1, are presented in Table 5.4b.

a)



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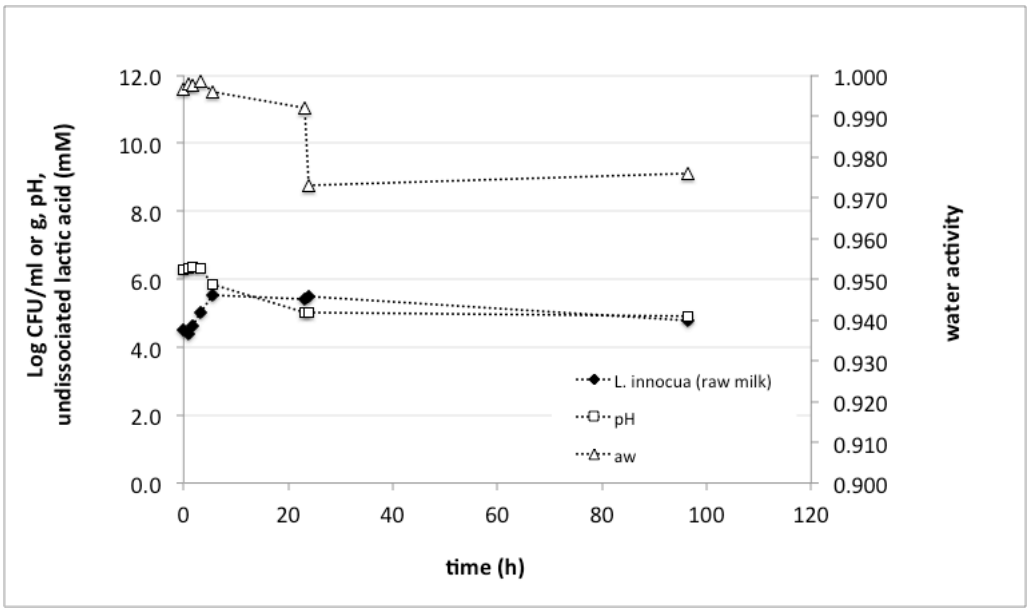
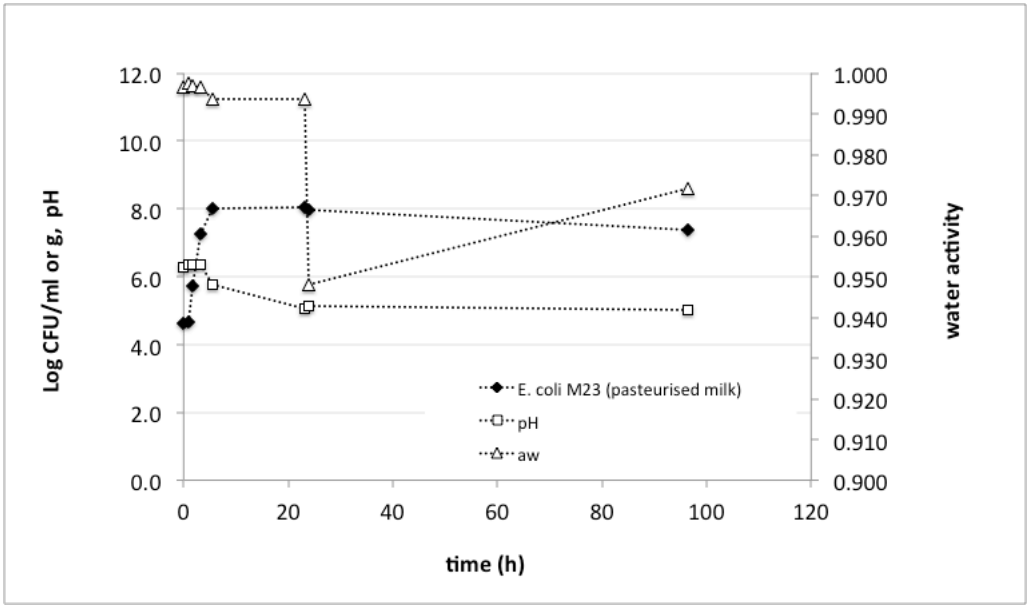


Figure 5.4a, b Changes in pH, water activity and undissociated lactic acid concentration in double-cream Brie-style cheese made from raw milk. Figure 5.4a (upper) also shows changes in levels of *E. coli* M23 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.4b (lower) show**s c**hanges in levels of *L. innocua* inoculated into the milk at the same time as addition of starter cultures.

c)



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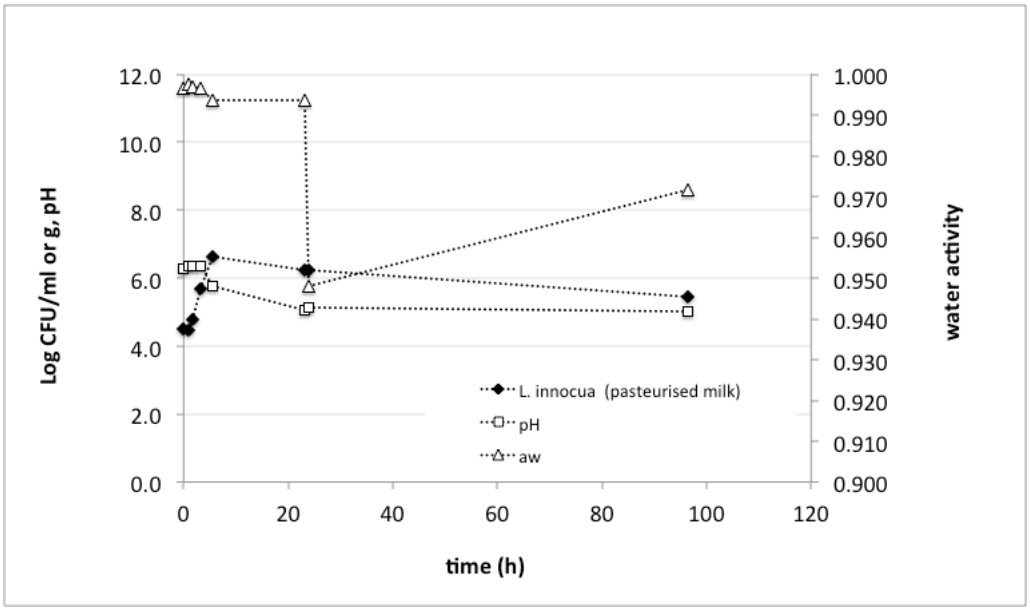
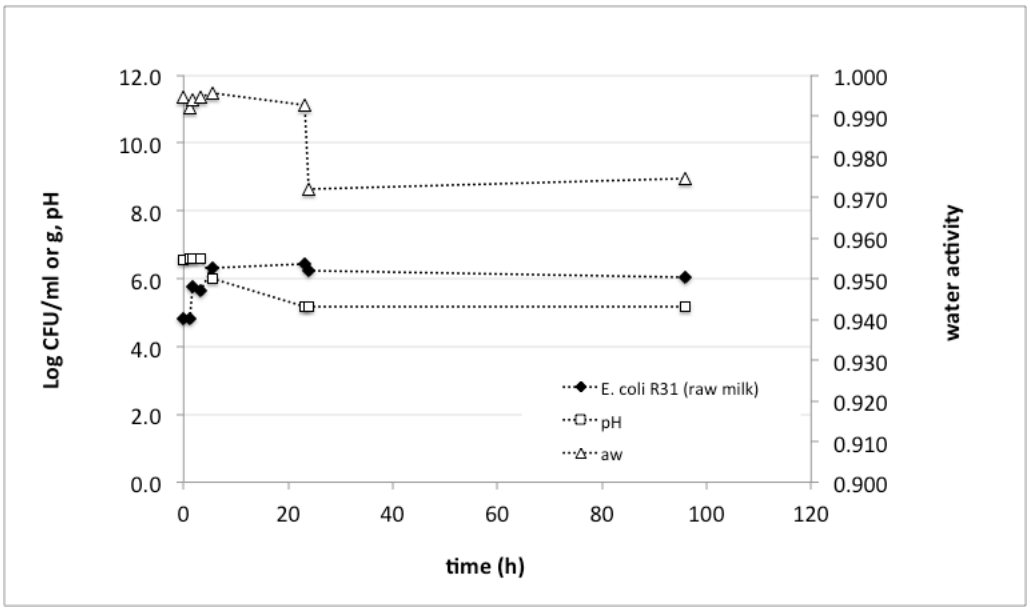


Figures 5.4c, d. Changes in pH, water activity and undissociated lactic acid concentration in double-cream Brie-style cheese made from pasteurised milk. Figure 5.4c (upper) also shows changes in levels of *E. coli* M23 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.4d (lower) shows changes in levels of *L. innocua* inoculated into the milk at the same time as addition of starter cultures.

e)



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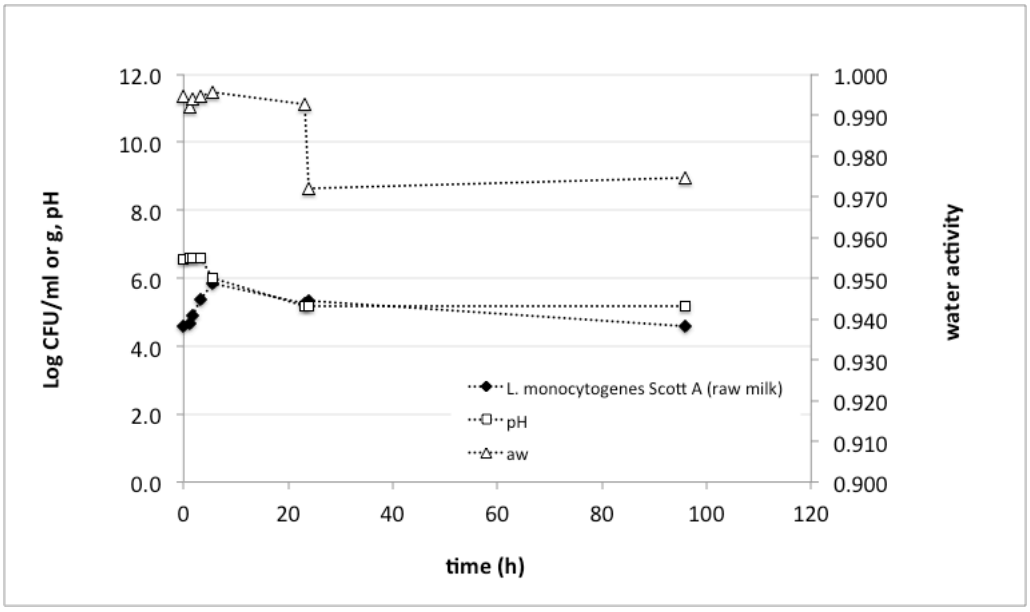
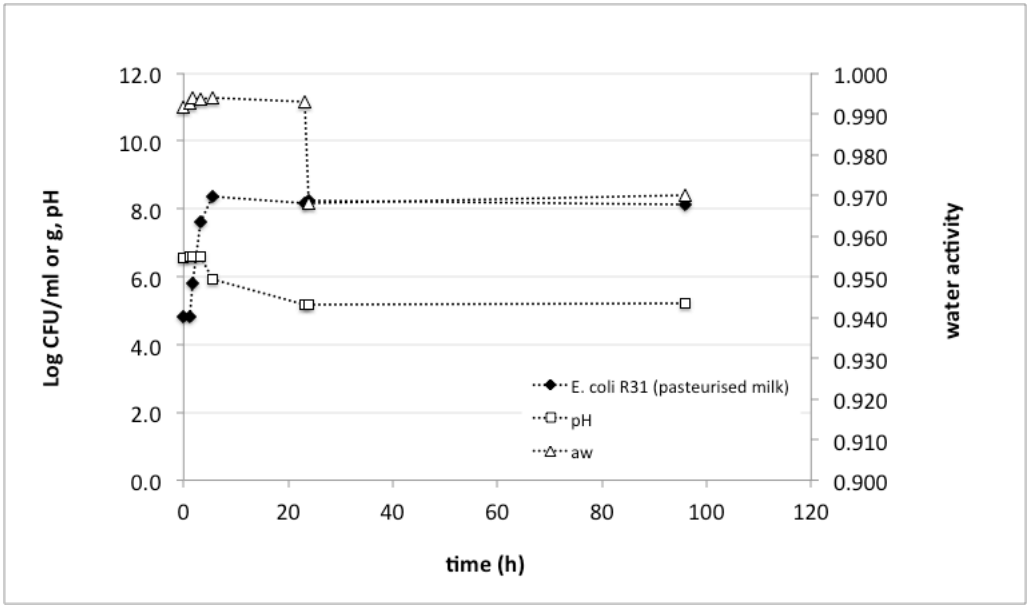


Figures 5.4e, f. Changes in pH, water activity and undissociated lactic acid concentration in double-cream Brie-style cheese made from raw milk. Figure 5.4e (upper) also shows changes in levels of *E. coli* R31 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.4f (lower) shows changes in levels of *L. monocytogenes* Scott A inoculated into the milk at the same time as addition of starter cultures.

g)



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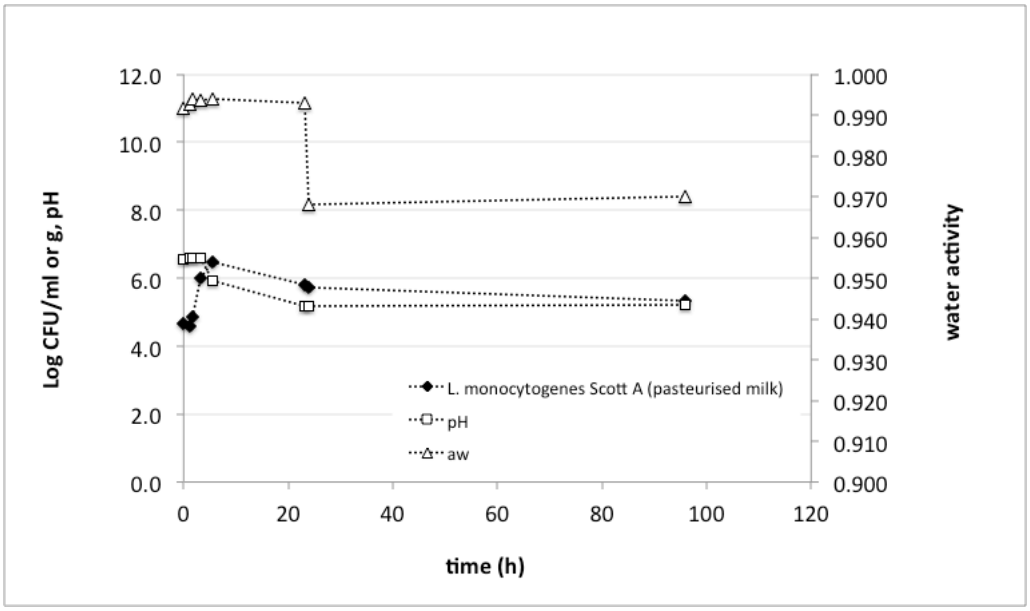


Figures 5.4g, h. Changes in pH, water activity and undissociated lactic acid concentration in double-cream Brie-style cheese made from pasteurised milk. Figure 5.4g (upper) also shows changes in levels of *E. coli* R31 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.4h (lower) shows changes in levels of *L. monocytogenes* Scott A inoculated into the milk at the same time as addition of starter cultures.

Table. 5.4a Summary data for changes in challenge organism levels and physico-chemical changes in double-cream Brie-style cheeses during fermentation/curd formation

Double-cream Brie	*E. coli* increase (log_{10}CFU)		*Listeria* ssp. increase (log_{10}CFU)		max. pH change	Time taken (h)	"Final" pH	"Final" a_w
Batch	M23	R31	*mono-cytogenes*	*innocua*				
Raw 1	1.22			0.98	1.45	~10	7.17	0.971
Raw 2		1.62	1.26		1.43	~10	7.90	0.970
Pasteurised 1	3.36			1.71	1.36	~10	7.34	0.964
Pasteurised 2		3.35	1.05		1.44	~10	7.91	0.966
Log difference pasteurised to raw	2.14	1.73	-0.21	0.73			Final lactic acid (%w/w)	0.02 *(but levels were much higher early during maturation
Average increase *Listeria*	**1.25**	*0.34*	(SD)					
Average increase *E .coli*	**2.39**	*1.14*	(SD)					
Incubation to curd processing:		> 4h x 38°C						
Starter culture:		types B, E						

Note that in these cheeses, the time taken for acidification to be completed was difficult to establish reliably due to the rate of acidification and times samples were taken and pH determined. Accordingly, the time to acidification was estimated from the data (*see* Figures 5.4a – h) by approximation by extrapolating the observed rate of acidification to the final pH achieved.

Table. 5.4b Generation time estimates for challenge organisms in double-cream Brie cheeses during fermentation/curd formation

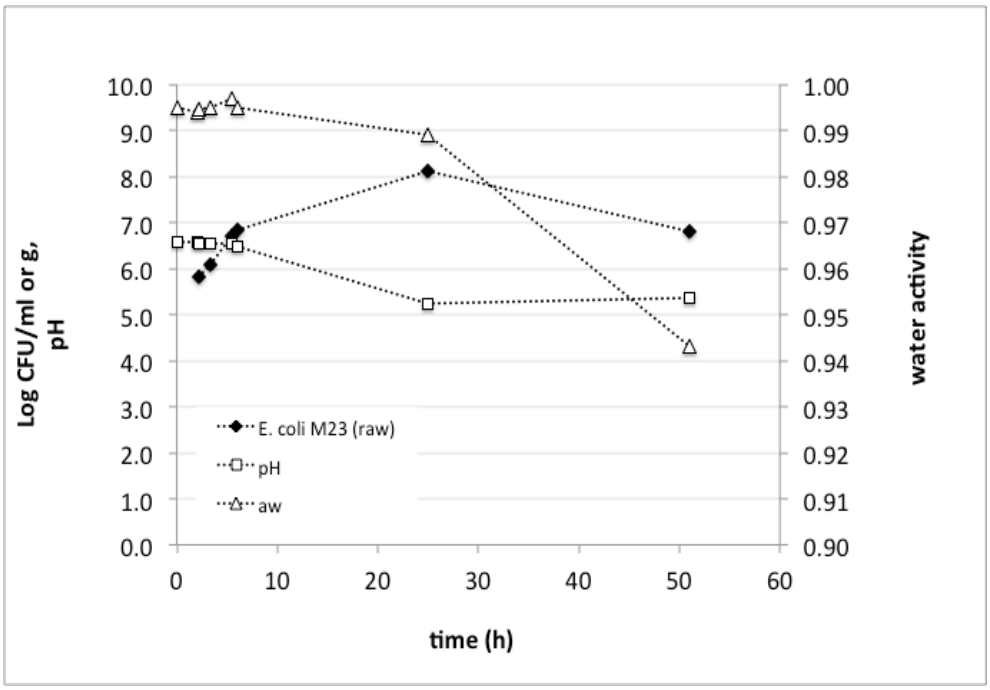
Double-cream Brie	Estimated *E. coli* gen time (min)		Estimated *Listeria* gen time (min)	
Batch	M23	R31	*L. monocytogenes*	*L. innocua*
Raw 1	50			66
Raw 2		30	33	
Pasteurised 1	60			70
Pasteurised 2		15	28	

As with the other challenge trials, the cessation of the growth of the challenge organisms corresponded with the increase in lactic acid levels and decline in pH rather than reduction in a_w. Growth in these cheeses seemed to be noticeably slower than for other cheeses. It is possible that the temperature employed (38°C) was super-optimal. Nonetheless, the total amount of growth during acidification was similar to other cheeses. In the preparation of double-cream Brie lower inoculum levels were used (to be able to measure growth of the challenge strains *cf*. all other trials in which higher inocula were used because inactivation was anticipated). This, however, also allows evaluation of the hypothesis that growth is inhibited by the challenge strains entering stationary phase. From the data in Figures 5.4, this suggestion in not supported and, instead, cessation of growth of the challenge organisms seems to be correlated with decline in pH and increase in lactic acid levels.

5.3.5 Wensleydale Cheese

Figures 5.5a – h present data describing changes in physico-chemical parameters during the processing of Wensleydale-style cheeses, up until the commencement of the maturation stage. The data are summarised in Table 5.5a. Four batches of Wensleydale cheeses were made: two from raw milk, and two from the same milk after pasteurisation. During the processing of the cheeses one strain of *E. coli* (either R31 or M23) and one species of *Listeria*, (either *L. monocytogenes* or its non-pathogenic analogue and surrogate, *L. innocua*) were inoculated into the milk at the same time as the starter culture was added. The data presented relate to the physico-chemical parameters, and microbiological changes, in each batch prior to them being separated and stored at different temperatures. For each strain/species of challenge organism in each batch produced, generation times were estimated from the data as described in Section 5.3.1 and the estimates presented in Table 5.5b.

a)



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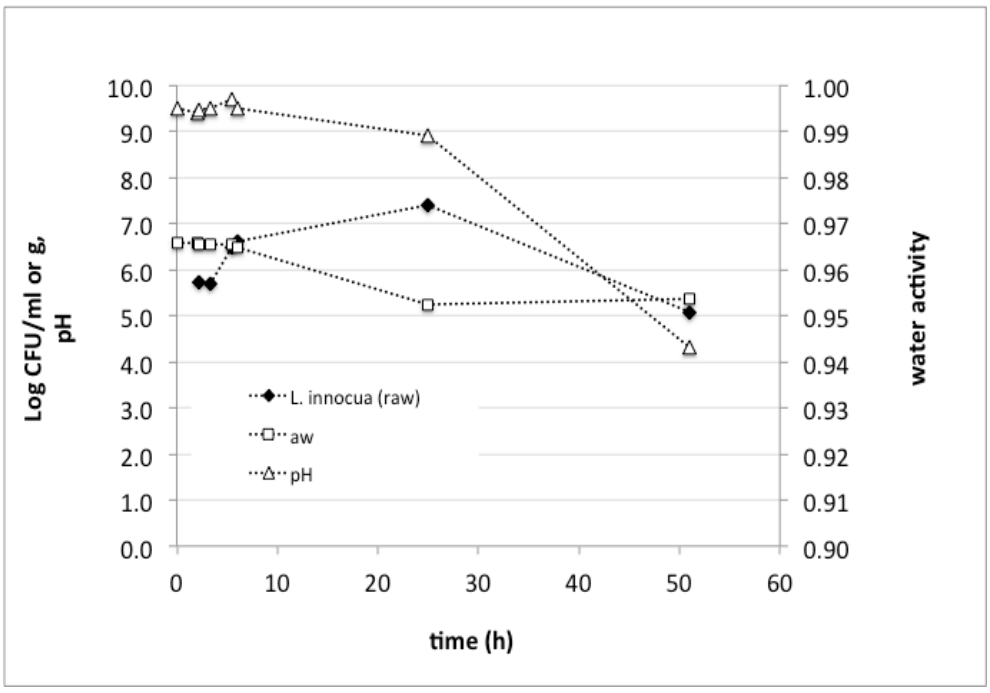
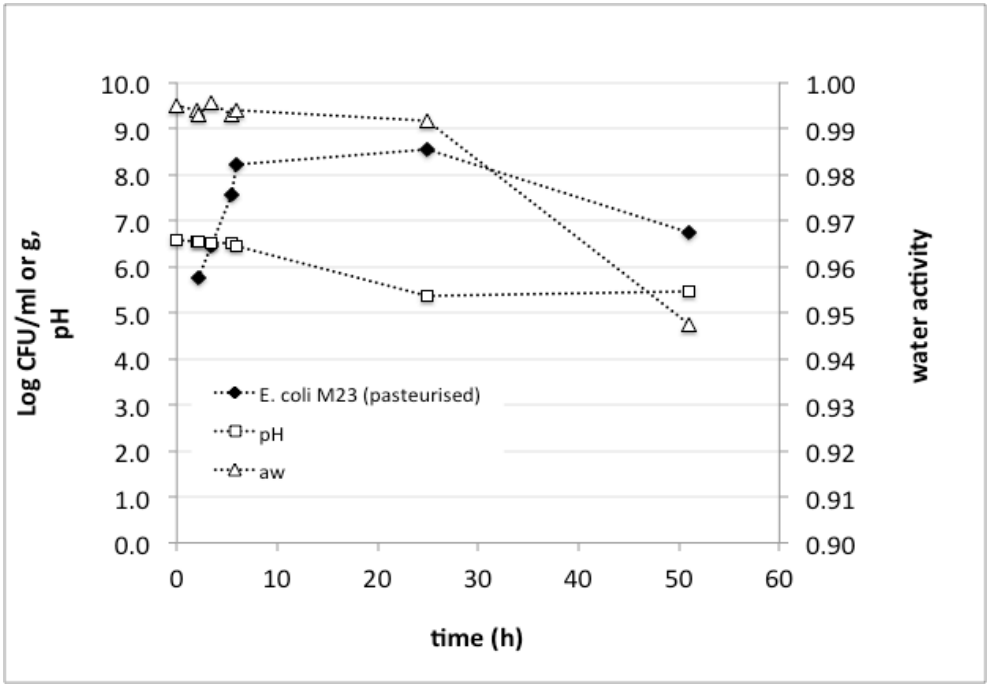


Figure 5.5a, b. Changes in pH and water activity in Wensleydale cheese made from raw milk.

Figure 5.5a (upper) also shows changes in levels of *E. coli* M23 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.5b (lower) shows changes in levels of *L. innocua* inoculated into the milk at the same time as addition of starter cultures.

c)



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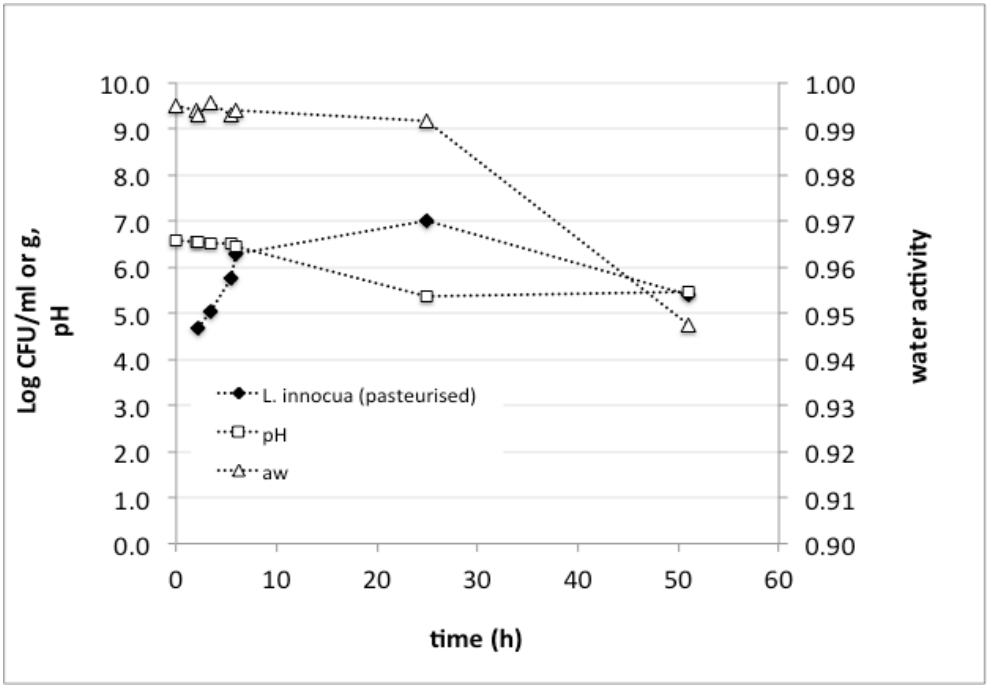

Figure 5.5c, d. Changes in pH and water activity in Wensleydale cheese made from pasteurised milk. Figure 5.5c (upper) also shows changes in levels of *E. coli* M23 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.5d (lower) shows changes in levels of *L. innocua* inoculated into the milk at the same time as addition of starter cultures.

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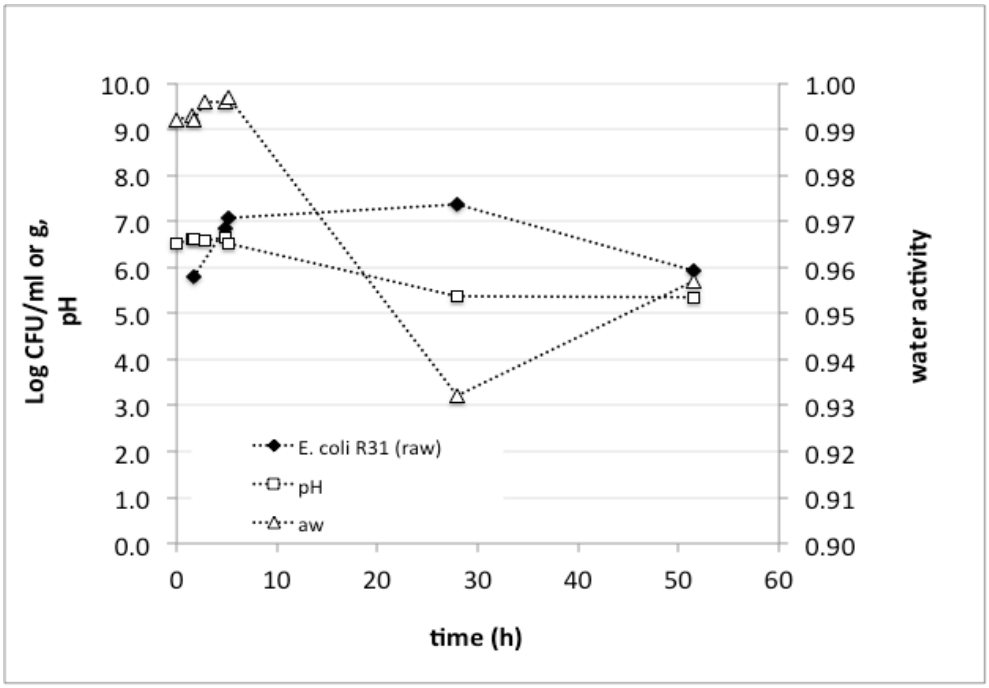

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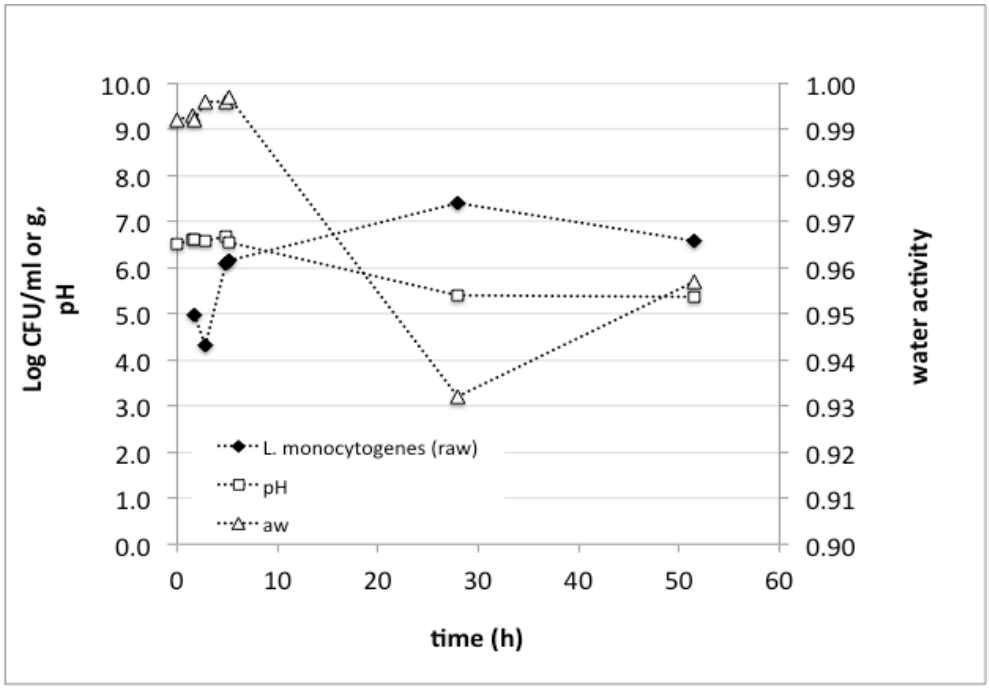
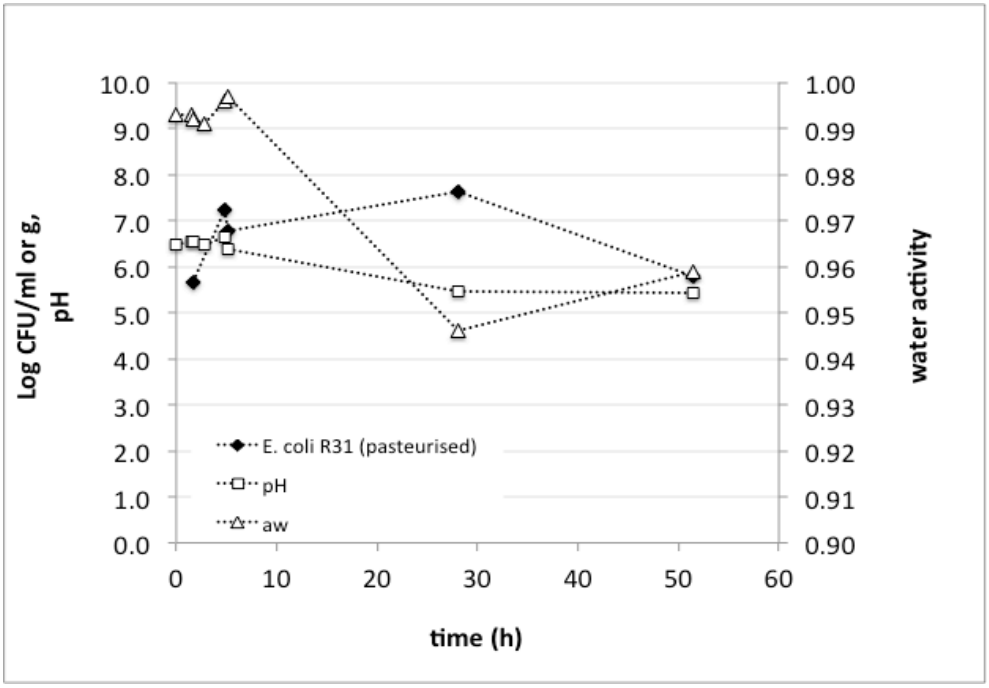


Figure 5.5e, f. Changes in pH and water activity in Wensleydale cheese made from raw milk.

Figure 5.5e (upper) also shows changes in levels of *E. coli* R31 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.5f (lower) show**s c**hanges in levels of *L. monocytogenes* inoculated into the milk at the same time as addition of starter cultures.

g)



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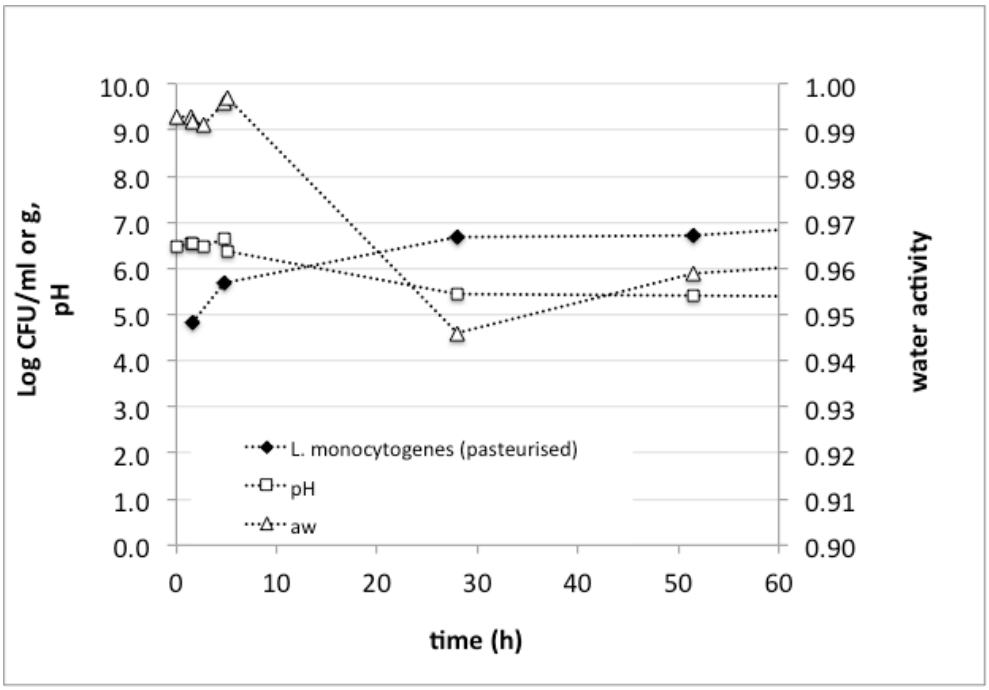


Figure 5.5g, h. Changes in pH and water activity in Wensleydale cheese made from pasteurised milk. Figure 5.5g (upper) also shows changes in levels of *E. coli* R31 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.5h (lower) shows changes in levels of *L. monocytogenes* inoculated into the milk at the same time as addition of starter cultures.

The challenge trial involving *L. monocytogenes* in Wensleydale cheese made from pasteurised milk was unusual compared to all other trials, because *L. monocytogenes* apparently continued to grow for up to 160 hours. Results from this trial are shown in greater detail in Figure 5.5i, below, with an extended time scale to reveal the 'unusual' growth response of *L. monocytogenes*. The growth was unexpected because *L. monocytogenes* was not predicted, from a range of predictive models, to be able to grow under the conditions of temperature, pH, a_w and lactic acid concentration prevalent during the challenge trial after 24 hours and because no growth after 24 hours was observed in the analogous Wensleydale cheese trials involving cheese made from raw milk, or *L. innocua* in Wensleydale cheese made from either raw or pasteurised milk. It is noted, however, that the aw data for this trial are also anomalous, and after 24 hours increases to levels higher than seen in other trials, and may explain the observed growth of *L. monocytogenes* in this trial.

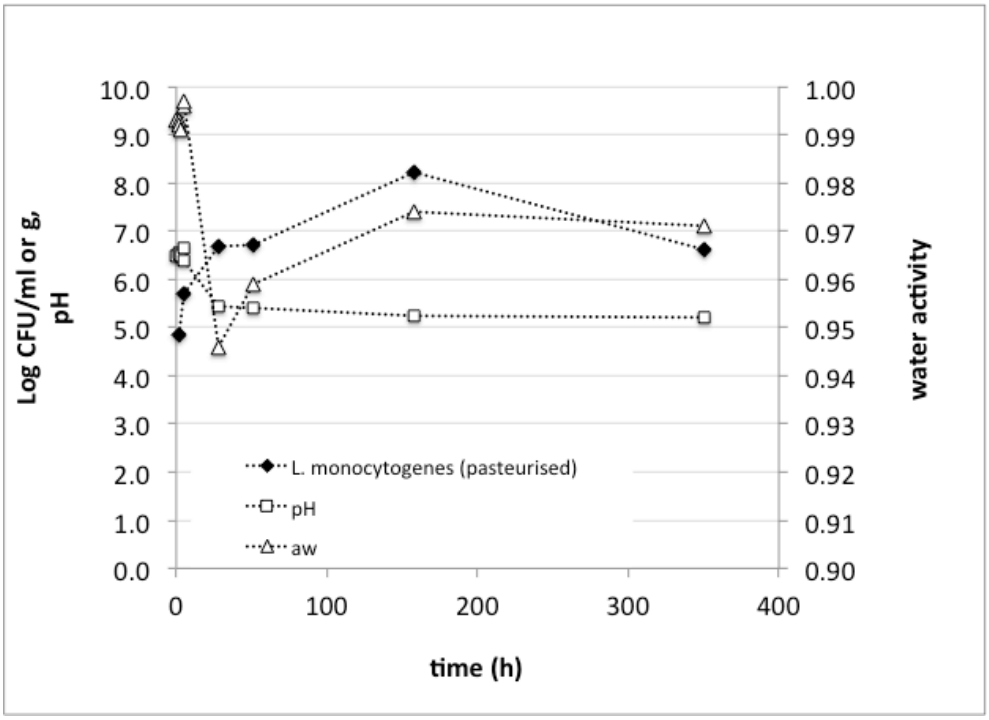


Figure 5.5i. Changes in *L. monocytogenes,* pH and water activity in Wensleydale cheese made from pasteurised milk. This is the same data as in Figure 5.5h, but with an expanded time scale to show the unusual behaviour of *L. monocytogenes* in this trial.

Summary data for the extent of growth of challenge organisms during challenge trials in Wensleydale-style cheese are shown in Table 5.5a, which also summarises the physico-chemical characteristics of the cheese. As with other trials, final pH and a_w of Wensleydale cheeses made were highly reproducible, irrespective of milk type (raw, or pasteurised) or challenge organisms introduced. In the Wensleydale trials, lactic acid levels measured were not consistent with typical levels in other cheeses, being a factor or ten or more greater than expected, or typical, of other

cheeses. Accordingly, the data are not shown. Table 5.5b presents generation time estimates for *Listeria* spp. and *E. coli* during curd formation in Wensleydale style cheese.

Table. 5.5a Summary data for changes in challenge organism levels and physico-chemical changes in Wensleydale cheeses during fermentation/curd formation

Wensleydale	*E. coli* increase (log_{10}CFU)		*Listeria* ssp. increase (log_{10}CFU)		pH change	Time taken (h)	"Final" pH	"Final" a_w
Batch	M23	R31	*mono-cytogenes*	*innocua*				
Raw 1	2.28			1.68	1.32	>6, <25	~5.40	~0.966
Raw 2		1.59	2.42		1.49	>6, <28	~5.28	~0.967
Pasteurised 1	2.80			2.52	1.35	>6, <25	~5.30	~0.965
Pasteurised 2		2.09	3.40* (1.97)		1.44	>6, <28	~5.36	~0.966
Log difference pasteurised to raw	0.52	0.50	0.98	0.84				
Average increase *Listeria*	2.51* (2.15)	*0.70* (0.39)*	*(SD)*					
Average increase *E .coli*	2.19	*0.50*	*(SD)*					

Incubation to curd processing: 1 h x 32°C, curd then 3.5 h x 38°C
Starter culture: type A

* These results include the full apparent increase in *L. monocytogenes* in the challenge trial in pasteurised milk Wensleydale cheese, despite that those results appear to be anomalous.

Table. 5.5b Generation time estimates for challenge organism in Wensleydale cheeses during fermentation/curd formation

Cheddar	Estimated *E. coli* gen time (min)		Estimated *Listeria* gen time (min)	
Batch	M23	R31	*L. monocytogenes*	*L. innocua*
Raw 1	62			130
Raw 2		23	56	
Pasteurised 1	29			28
Pasteurised 2		36	33	

As with the other challenge trials, the cessation of the growth of the challenge organisms corresponded with the increase in lactic acid levels and decline in pH rather than reduction in a_w. Several observed growth rates are slower than expected from the predictive models described earlier, based on the temperature and physico-chemical conditions in the milk/cheese, and the growth rates in these trials were not as reproducible between analogous trials as observed in trials involving other types of cheese.

It is possible that the temperature employed in processing of Wensleydale cheese (38°C) was super-optimal. Nonetheless, the total amount of growth during acidification was similar to other cheeses. In the preparation of Wensleydale challenge trials somewhat lower inoculum levels were used than in the Cheddar, Gouda and Feta trials and were more consistent with the levels proposed by Ross (2011) of ~10^5 CFU/ml^{-1}. From the data in Figures 5.5, the suggestion that the growth observed during challenge trials is limited by the population of the challenge organism entering stationary phase is, apparently, not supported. However, while cessation of growth of the challenge organisms seemed to be correlated with decline in pH and increase in lactic acid levels in all other trials, cessation of growth of the challenge organisms was not as closely correlated with the decline in pH. Equally, the rate of acidification in the Wensleydale cheeses was the slowest of all cheeses made despite that Wensleydale used the same starter culture (Type A) as used for Cheddar and Feta trials, and the incubation temperatures were similar. That the rate of acidification is slower suggests that the starter culture also was growing more slowly. Wensleydale was the only cheese made using goat's milk. From the data and observations described, it is possible that growth potential of *E. coli*, *Listeria* spp. and starter cultures is reduced in goats milk, compared to cow's milk. This suggestion warrants further investigation.

5.3.7 Viable count increases during acidification

Table 5.6 compares the maximum increase of each challenge organism in raw milk and pasteurised milk for each style of cheese produced. It should be noted that the increase might be due to a combination of concentration of challenge organisms into the curd in addition growth. While concentration would he expected to occur at the time of curd formation, Figure 1- 5 show that cell density increases often occurred relatively rapidly and steadily after addition of challenge strains. Also, typically ~90% of the milk is whey, leading to an expected ten-fold increase in cell density per gram of curd/cheese, compared to the cell concentration in the milk (Maher *et al*., 2001). Maher *et al.* (2001), however, specifically distinguished entrapment from growth and, in a smear cheese, reported that concentration into the curd by entrapment represented only 0.6 $\log_{10}$cfu increase. They suggested that this lower-than-reported value was a reflection of the high moisture content of the cheese. In almost all trials, increases in pathogen concentrations were greater than ten-fold, suggesting that some pathogen growth occurred during acidification in all trials.

The data accentuate that in almost all cases the increase of the challenge organism is slightly greater (given that starting inocula were identical) in the pasteurised milks. The difference in growth was usually greater for *E. coli* than for *Listeria* spp. This observation supports speculation (earlier in this report) that unpasteurised milk is more inhibitory to microbial growth but it must be noted that the

difference is small compared to the amount of growth observed. Schvartzman *et al.* (2011) also reported that more growth of *L. monocytogenes* was observed in a smeared cheese made with pasteurised milk than an equivalent cheese made from raw milk.

Of equal, or perhaps greater, relevance than the maximum population density achieved by each challenge organism in each trial, is the *increase* in cell concentration that was observed. In addition to simple concentration this increase could also be a function of the growth rate of the cell, the rate of acidification, and the consequence of competition for nutrients (e.g., induction of stationary phase responses as discussed earlier). To investigate this further, the increases in Log_{10}CFU for each challenge organism for each challenge trial were estimated and collated, and are presented in Table 5.7. Recognising that direct comparisons of growth rates is confounded by differences in cheeses, temperature, inoculum levels etc. the log_{10}CFU increase for each organism in each cheese was compared to the average growth observed for that organism (*e.g*., all *E. coli* or all *Listeria* spp.) in each cheese style to generate a ratio that could be compared across raw or pasteurised milk cheese and by species of *Listeria* or strain of *E. coli*.

The results, although not consistently highly (i.e. >95%) statistically significant, reinforce that more growth was observed during acidification of pasteurised milk cheeses, irrespective of species or strain. Specific exceptions to this general response were *L. monocytogenes* in Brie (in which more growth was observed in raw milk) and *L. innocua* and *E. coli* R31 in Feta Furthermore, *E. coli* R31 grew more prolifically than *E. coli* M23, whereas the two *Listeria* species showed more similar amounts of growth. Equally, *E. coli s*trains grew more prolifically than *Listeria* spp., also reinforcing the observations from the individual cheese challenge trial data, and consistent with the known faster growth rate of *E. coli* that *L. monocytogenes* or *L. innocua.*

Table. 5.6 Summary data for maximum levels of challenge organisms in different cheeses made in this study prior to the commencement of the maturation (ripening) stage

	***E. coli* M23**		***L. innocua***		***E. coli* R31**		***L. monocytogenes***	
	(raw)	(past'd)	(raw)	(past'd)	(raw)	(past'd)	(raw)	*(past'd)*
Cheddar	8.37	8.64	7.95	8.09	8.82	8.95	7.55	7.46
Gouda	8.47	8.75	8.16	7.91	9.03	9.16	7.18	7.42
Feta	8.86	9.25	8.49	8.65	9.25	9.36	8.34	8.46
Wensleydale	8.1	8.54	7.4	7.18	7.37	7.77	7.38	8.23
Brie	5.38	8.03	5.51	6.62	6.42	8.35	5.85	6.48

Table. 5.7 Summary data for increase in levels of challenge organisms in different cheeses made in this study prior to the commencement of the maturation (ripening) stage

	Escherichia coli				*Listeria spp.*			
	M23		R31		*innocua*		*monocytogenes*	
	(raw)	(past.)	(raw)	(past.)	(raw)	(past.)	(raw)	(past.)
Absolute increase (log_{10}CFU)								
Cheddar	1.65	1.85	2.00	2.12	1.45	1.65	1.06	1.17
Gouda	1.89	2.11	2.24	2.32	1.17	1.69	1.29	1.35
Feta	2.28	2.48	2.55	2.43	1.89	1.88	1.89	1.97
Wensleydale	2.28	2.80	1.59	2.09	1.68	2.52	2.42	1.97
Brie	1.22	3.36	1.62	3.35	0.98	1.71	1.26	1.05
Increase in each cheese relative to average increase (by species/strain)								
Cheddar	0.87	0.97	1.05	1.11	1.09	1.24	0.80	0.88
Gouda	0.88	0.99	1.05	1.08	0.85	1.23	0.94	0.98
Feta	0.94	1.02	1.05	1.00	0.99	0.99	0.99	1.03
Wensleydale	0.90	1.13	0.93	1.04	0.89	1.16	0.96	-
Brie	0.57	1.10	0.76	1.57	0.78	1.37	1.01	0.84
Mean increase relative to average for genus (Standard Deviation)	0.83 *0.15*	1.04 *0.07*	0.97 *0.13*	1.16 *0.23*	0.92 *0.12*	1.20 *0.14*	0.94 *0.08*	0.93 *0.09*
significance		*p<0.05*		*p < 0.2*		*p < 0.01*		*n.s.*

5.3.8 Is the cessation of growth of pathogens governed by acidification, or commencement of stationary phase?

In interpreting the population dynamics of the challenge of the organisms, particularly when most challenge trials used relatively high inoculum densities it is pertinent to ask whether the cessation of growth is due to the action of the starter cultures in generating lactic acid and reducing pH, and salting/brining processes, etc. or whether the population density of the challenge organisms became self-limiting e.g., due to exhaustion of a key nutrient. If the challenge organisms were self-limited, it might be expected that the extent of growth observed would be *less* for higher inoculum densities, i.e. that a negative correlation between inoculum density and extent of growth would be observed.

To explore further this possibility, the relationship between inoculum density and amount of growth was investigated by plotting the amount of growth observed for each organism in each trial against the inoculum density used. The results are presented in Figure 5.6a for *E. coli* data and Figure 5.6b for *Listeria* spp. data.

Inoculum densities varied from ~4 to ~7 $\log_{10}$CFU/ml of milk. The extent of growth observed showed no systematic effect of inoculum density, either for *E. coli* or *L. monocytogenes*, suggesting that the cessation of growth in most cases was probably due to the acidification of the milk due to starter culture activity leading to conditions that were inimical to growth of the challenge organisms, although the onset of stationary phase of the challenge organisms may also have been involved. Figures 5.6a, b further reinforce that more growth of *E. coli* is possible during the acidification process, with average increases of ~2.2 $\log_{10}$CFU compared to average increases in *Listeria* spp. during acidification of ~1.6 $\log_{10}$CFU.

While the evidence presented is not unequivocal, the data in Figures 5.6 strongly suggest that the extent of growth observed is limited by the action of the starter culture, acidification and salting/brining steps.

5.3.9 Is *rate* of acidification important?

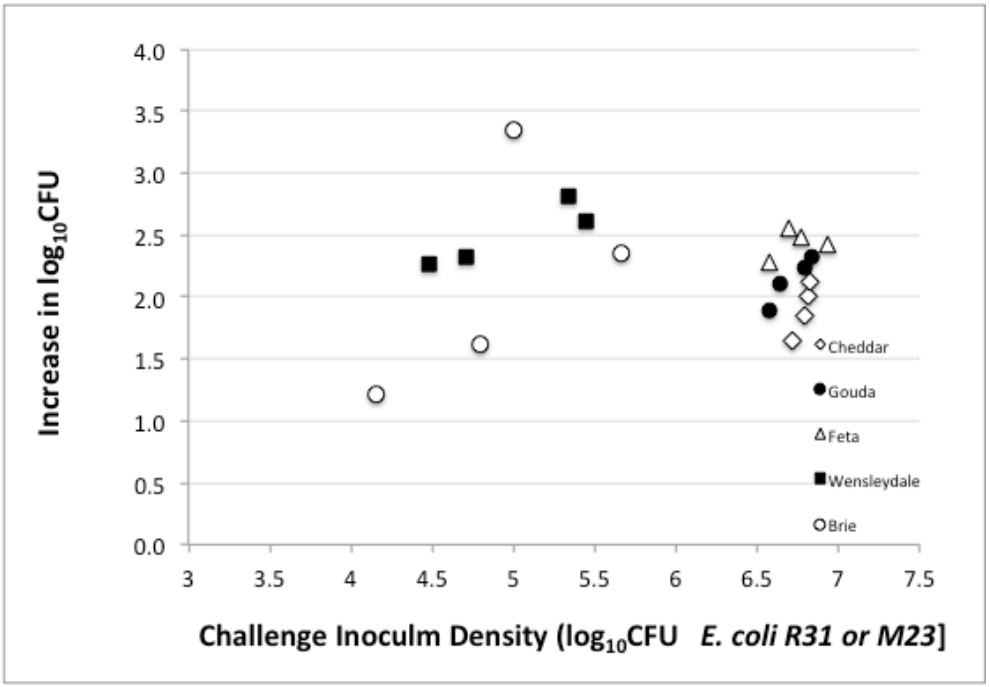
Given the results of described in Section 5.3.8, a further analysis was undertaken to evaluate the relationship between the time taken for the cheese to reach its final pH, and the extent of growth observed. The time for final pH for each cheese style was estimated empirically by extrapolation of the changing pH of the milk over time, based on the data presented in Figures 5.1 to 5.5. In most cases, the final pH was achieved at some time between observations at ~6 h after addition of starter cultures, and the next set of observations at ~24 h after addition of starter cultures and challenge organisms. In several cases (particularly for Wensleydale cheese), the time to achieve final pH had to be estimated empirically from the limited data describing the rate of decline of pH after addition of the starter culture. The following times (h) were estimated as the time taken to achieve final pH for each cheese:

Cheddar	5
Gouda	10
Feta	8
Double-cream Brie	10
Wensleydale	15

From the estimate of the time taken to achieve final pH, the rate of acidification was estimated from the change in pH from initial pH of the milk to final pH in the curd, divided by the estimate of the time taken for the final pH to be achieved (above). Given the limitations in the data, the assessment of the significance of the rate of acidification is not statistically rigorous, but was undertaken nonetheless in an attempt to begin to resolve whether rate of acidification would be a useful predictor of potential growth of pathogens were they present in the milk used for cheese making.

Figure 5.7a shows correlations between changes in challenge organism densities and times to final pH whereas Figure 5.7b shows correlations between changes in challenge organism densities and rate of acidification in the various cheeses produced. From the Figures it is suggested that, in most cases, increased time to acidification permits increased growth of pathogens. Similarly, less growth of pathogens is observed, generally, when acidification rates are faster. Correlation coefficients were calculated for each organisms and each independent variable (time to final pH or rate of acidification) and in most cases were less than 0.5, suggesting that while the rate of acidification

a)



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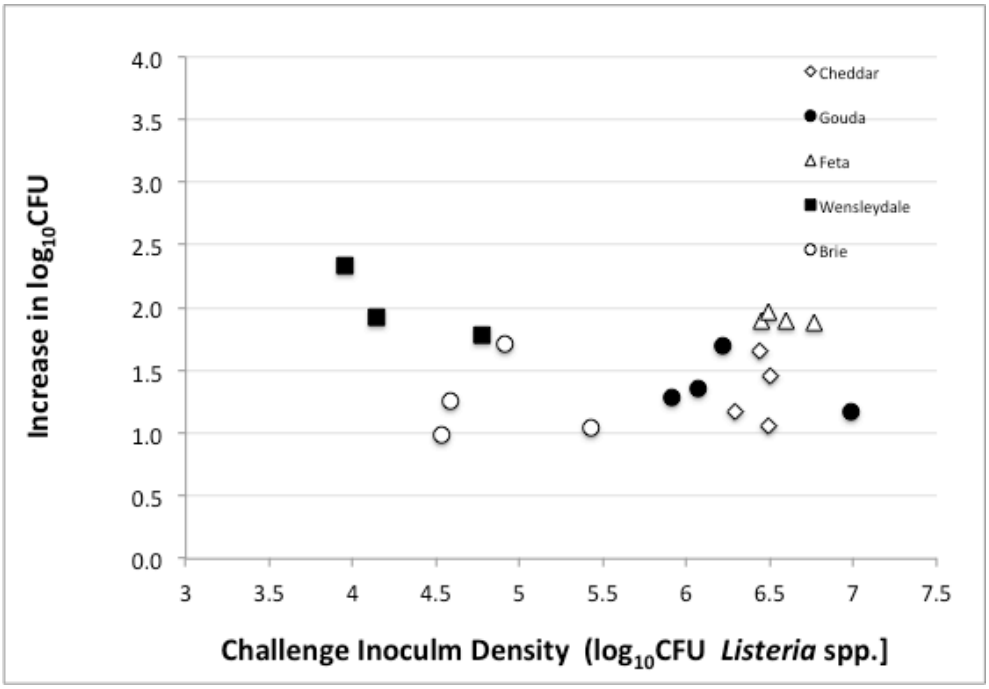
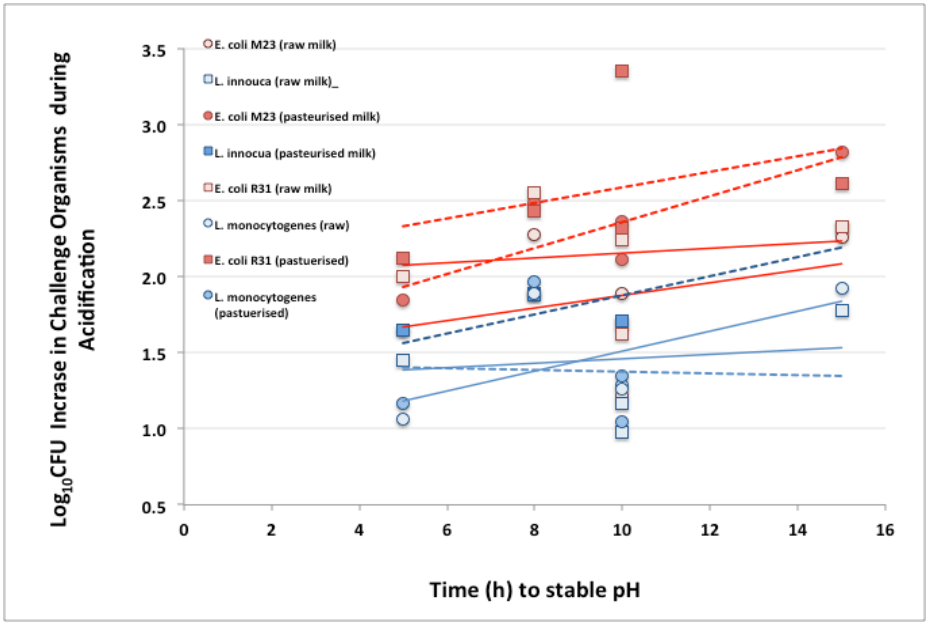


Figures 5.6a, b. Correlations between inoculum densities of challenge strains in all trials and the extent of growth observed. Figure 5.6a (upper) also shows changes in levels of both strains of *E. coli* while Figure 5.6b (lower) shows changes in levels of both species of *Listeria*.

a)



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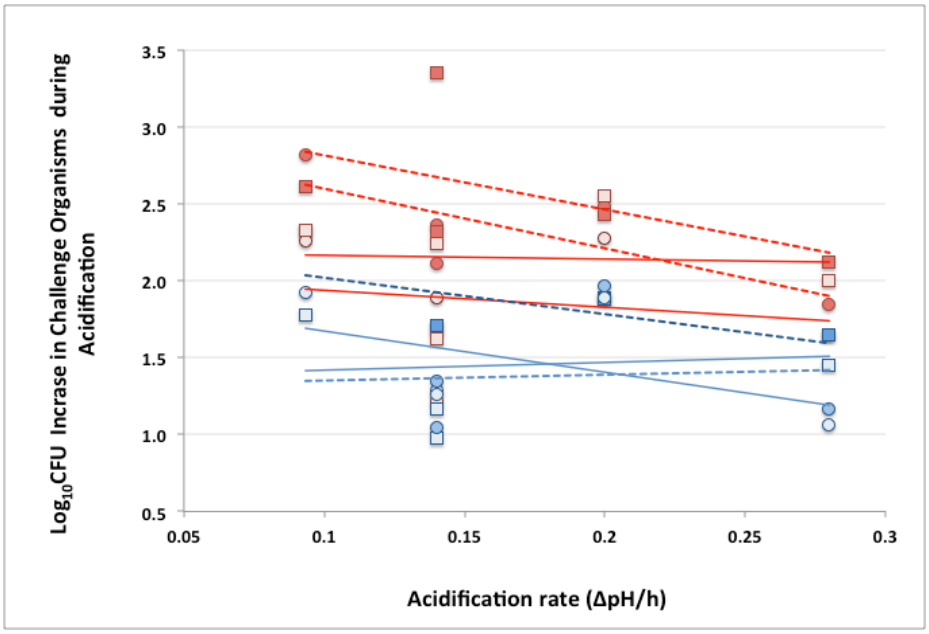


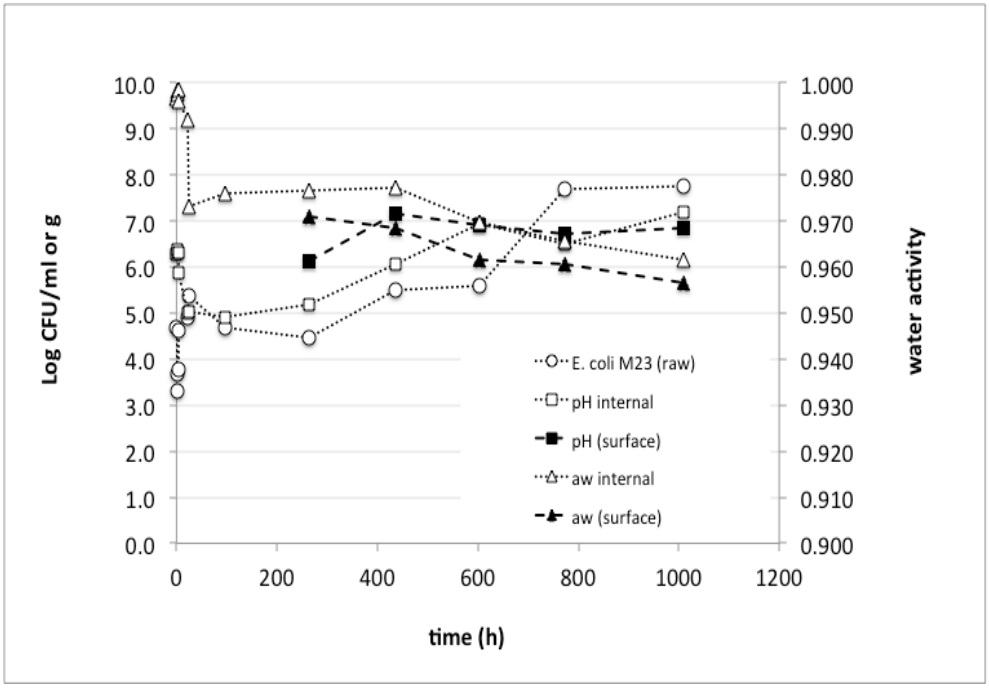
Figures 5.7a, b. Correlations between time to, or rate of, acidification of cheese and potential for pathogen growth. Figure 5.7a (upper) shows the extent of growth of each challenge organism as a function of the time after addition of the starter cultures before the final pH of the cheese was achieved, while Figure 5.7b (lower) shows changes as function of the *rate* of acidification of the cheeses. In both Figures, data for *E. coli* are shown using red plot symbols, and data for *Listeria* spp. as blue plot symbols. The legend in Figure 5.7a also relates to Figure 5.7b. Lines are regressions through the various data sets: dotted lines relate to pasteurised milk cheeses, solid lines relate to raw milk cheeses.

does affect the extent of growth of pathogens other, as yet undefined, factors also contribute variability to the response. The presentation of the data again reinforces that the potential for growth of *E. coli* is greater than that of *Listeria* spp. and that pasteurised milk cheeses tended to allow more growth of the challenge organisms than raw milk cheeses. From the data, and for reasons outlined above, there was no apparent difference in the time to achieve final pH or rate of acidification as a function of raw or pasteurised milk. Accordingly, it was not possible to resolve whether raw *vs.* pasteurised milk also affected the growth of starter cultures. However, there was no systematic difference in the final pH of analogous cheeses made from raw or pasteurised milk (*see* Tables 5.1a to 5.5a).

5.3.9 Changes in pH and water activity in surface mould-ripened cheeses

It is known that surface- and internal-mould and bacterial ripened cheeses undergo pH changes as ripening proceeds. These changes may alter the potential for pathogen growth and survival. As was demonstrated in Section 4.3, growth of both *Listeria* spp. and *E. coli* was observed in challenge trials of double-cream Brie style cheese after the formation of the curd as ripening progressed. Figures 5.8a–h present differences in pH and water activity at the surface, and internally, in the four challenge trials involving Brie-style cheeses. For each trial, the changes in the levels of the challenge organisms are also shown. The size and firmness of the cheeses made precluded challenge organism levels to be determined in both surface and core samples of those cheeses.

a)



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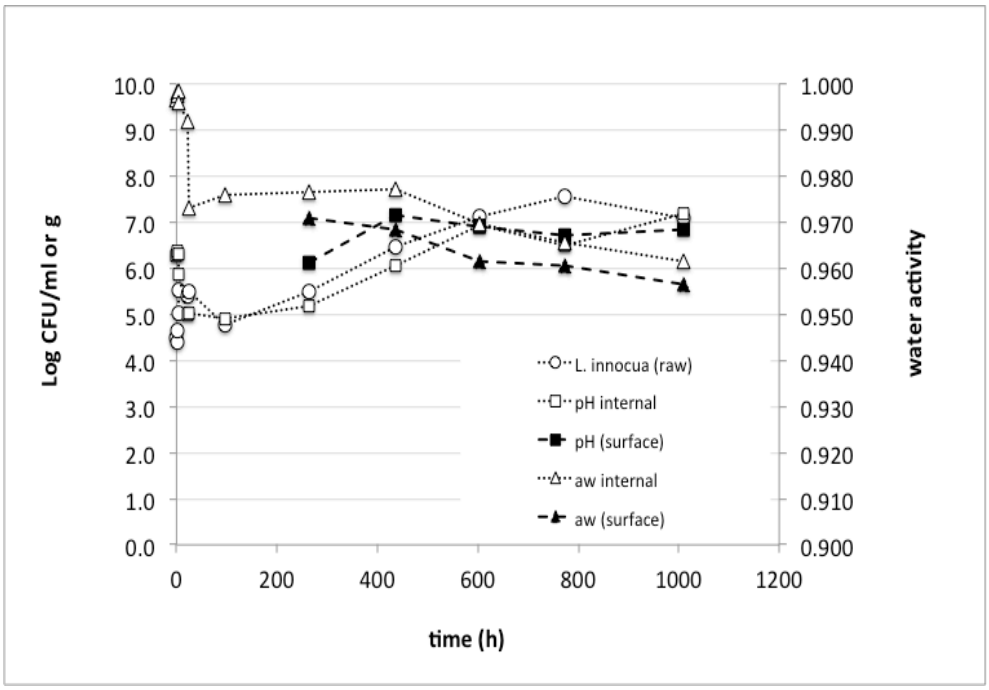
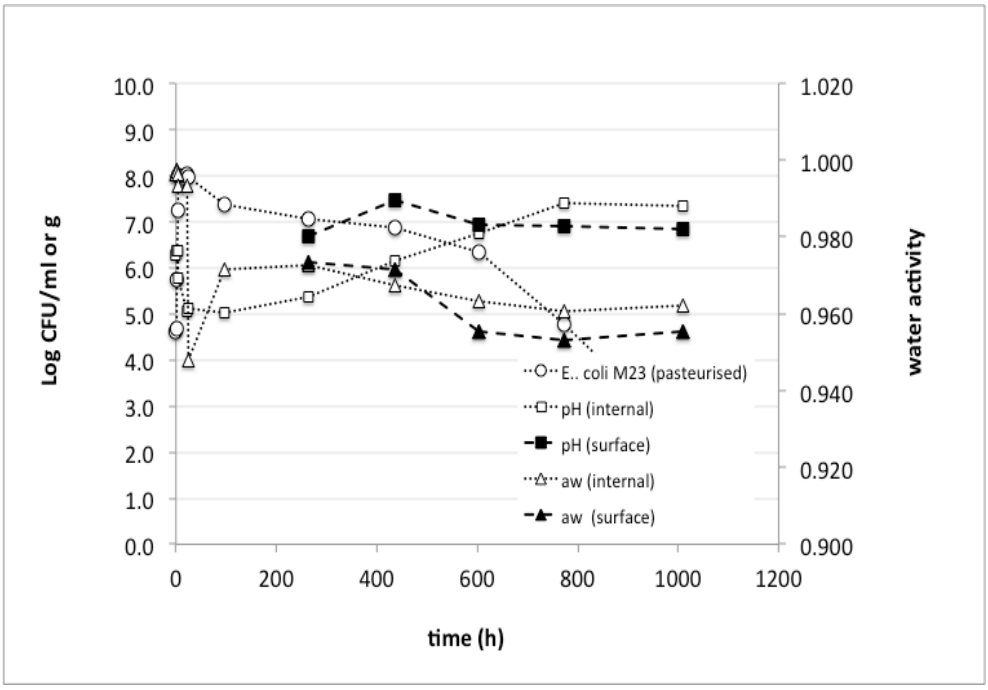
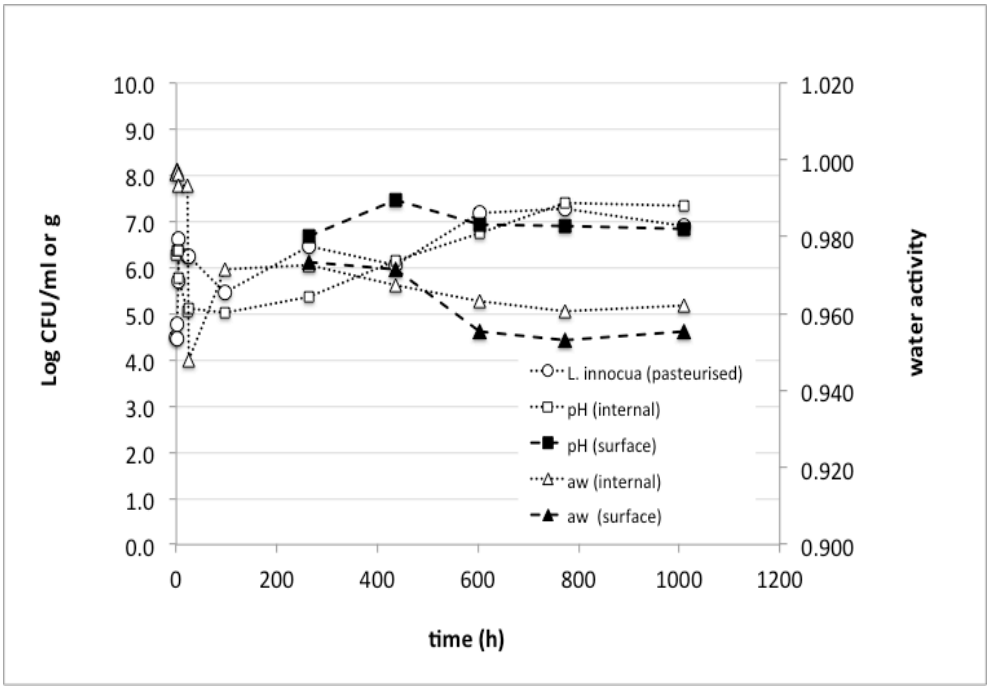


Figure 5.8a, b. Changes in pH, water activity and challenge organism levels in raw-milk double-cream Brie cheese and showing differences in surface and internal pH and water activity. Figure 5.7a (upper) shows changes in levels of *E. coli* M23, while Figure 5.7b (lower) show**s c**hanges in levels of *L. innocua* inoculated into the milk at the same time as addition of starter cultures.

c)

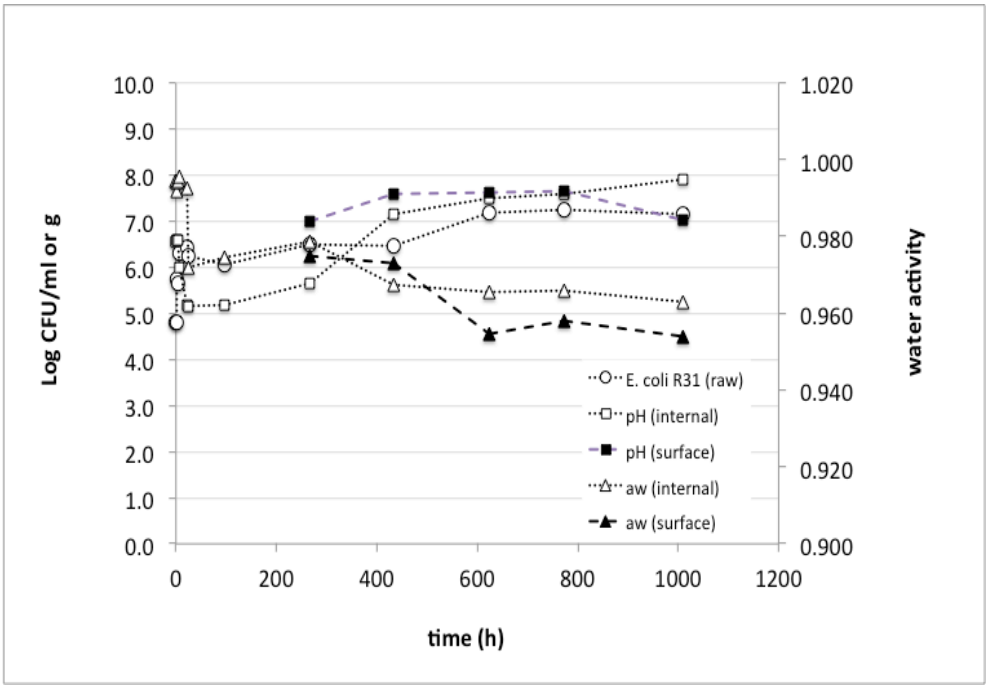


d)

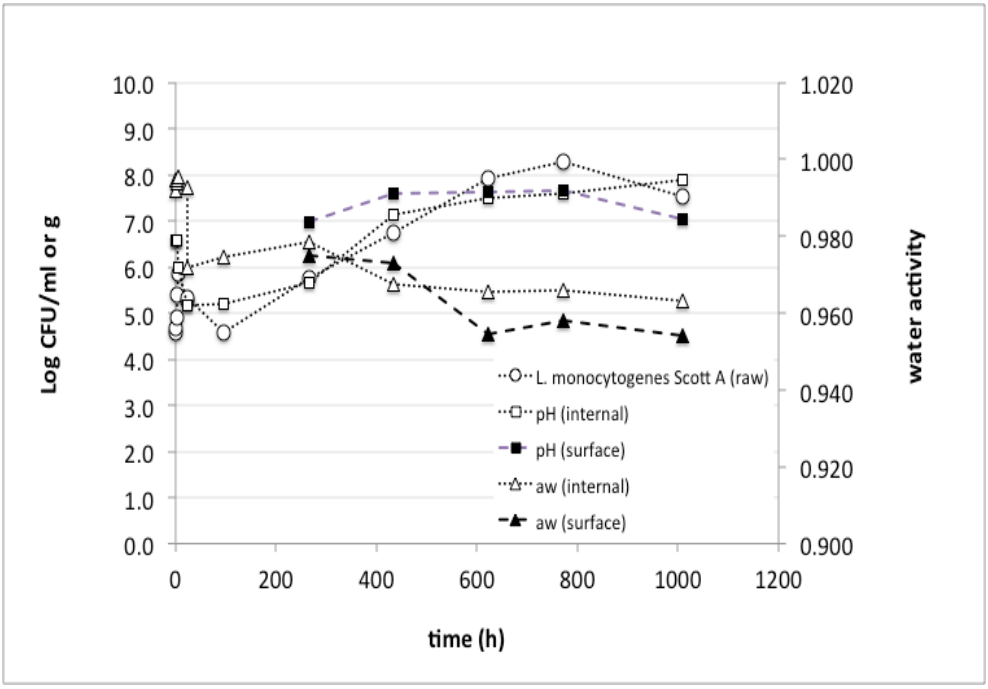


Figures 5.8c, d. Changes in pH, water activity and challenge organism levels in pasteurised milk double-cream Brie cheese and showing differences in surface and internal pH and water activity. Figure 5.7c (upper) also shows changes in levels of *E. coli* M23 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.7d (lower) show**s c**hanges in levels of *L. innocua* inoculated into the milk at the same time as addition of starter cultures.

e)

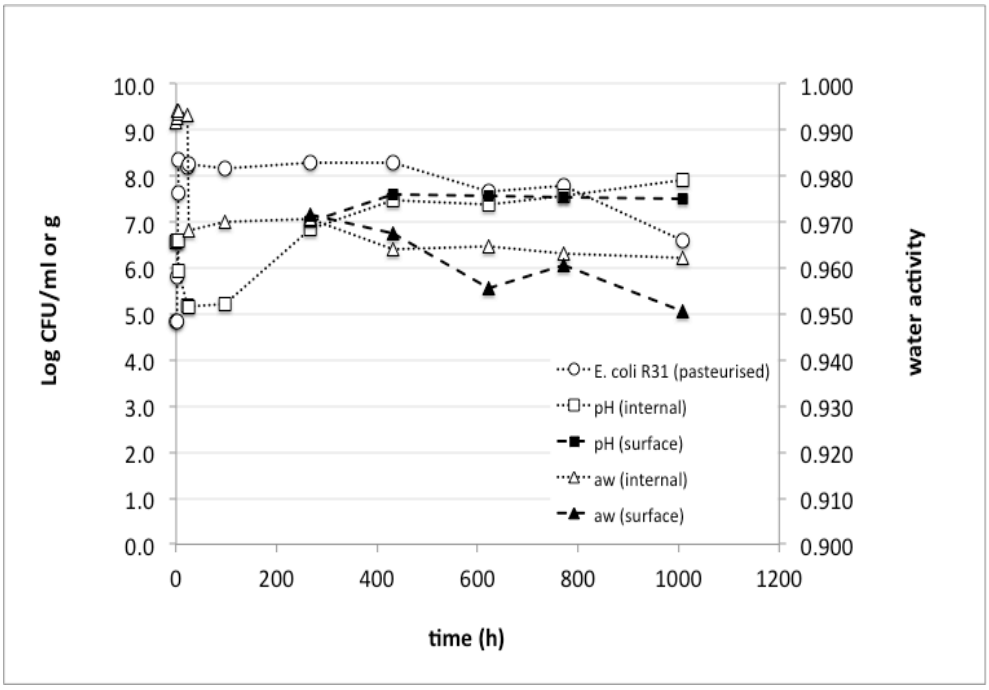


f)

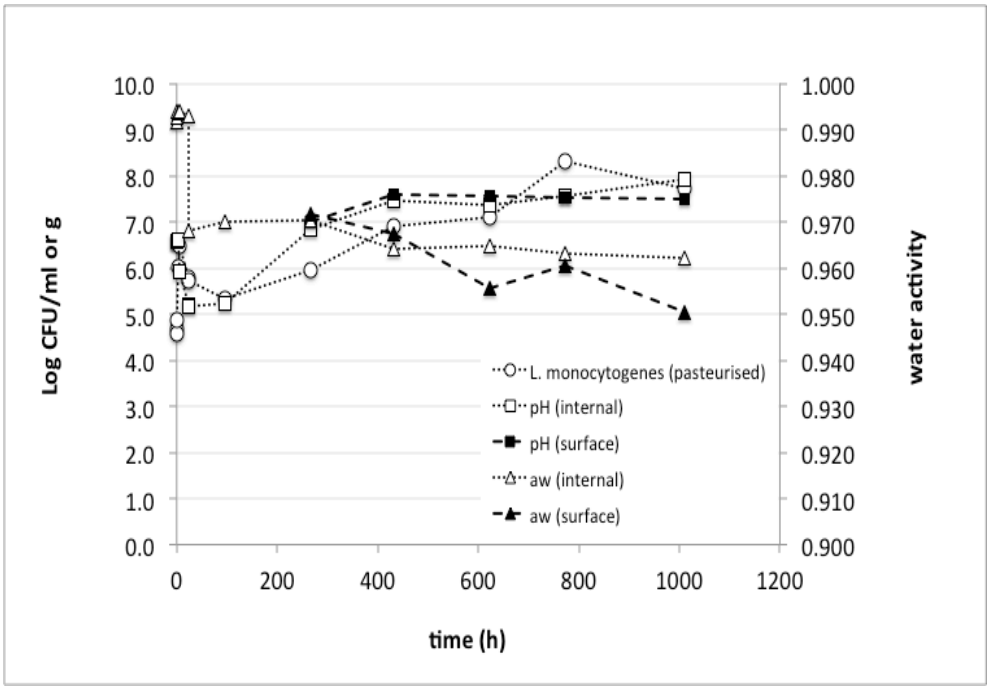


Figures 5.8e, f. Changes in pH, water activity and challenge organism levels in raw milk double-cream Brie cheese and showing differences in surface and internal pH and water activity. Figure 5.7e (upper) also shows changes in levels of *E. coli* R31 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.7f (lower) shows changes in levels of *L. monocytogenes* Scott A inoculated into the milk at the same time as addition of starter cultures.

g)



h)



Figures 5.8g, h. Changes in pH, water activity and challenge organism levels in pasteurised milk double-cream Brie cheese and showing differences in surface and internal pH and water activity. Figure 5.7g (upper) also shows changes in levels of *E. coli* R31 inoculated into the milk at the same time as addition of starter cultures, while Figure 5.7h (lower) show**s c**hanges in levels of *L. monocytogenes* Scott A inoculated into the milk at the same time as addition of starter cultures.

As expected, the pH of the surface, or 'rind' of the cheese begins to increase before the pH of the core, but the rate of change of pH in both is very similar. This is because the pH increase is due to the metabolism of the surface mould used in making of Brie, which involves proteolysis of cheese proteins, and the release of amine groups resulting from catabolism of amino acids. Amine groups are 'basic', and lead to an increase in pH. Differences were evident from about 200 to 600 hours after commencement of cheese-making. After 600 hours the pH in the core was effectively the same as the pH of the core. During the period of differences, the pH at the surface was up to 1 to 1.5 pH units higher than the core.

Water activity differences between surface and core were also assessed but differences were less pronounced than the pH differences. In general, a_w at the surface of the cheeses was slightly lower than the core, as might be expected due to greater loss of water from the surface of the cheese, compared to the core. Nonetheless, those differences might be expected to offset, to some extent, the effect of the higher surface pH on potential for pathogen growth.

5.3.10 Evaluation of Growth/No Growth Models

To assess the reliability of existing predictive microbiology models for evaluation of pathogen growth *potential* in cheeses, the physico-chemical data measured, or derived from measurements made on the cheeses during the challenge trials were tabulated and, as appropriate, translated into units of measurement appropriate to the various models available. The data are tabulated in Appendix 9. Growth rate predictions, where appropriate, were also made.

The models chosen for evaluation are:

i) *Listeria monocytogenes* growth rate model of Mejlholm and Dalgaard (2007)
ii) *Listeria monocytogenes* growth probability model of Mejlholm and Dalgaard (2007)
iii) Unpublished growth rate model for *L. monocytogenes* developed at University of Tasmania based on data of Tienungoon (1998; *see* Appendix 10)
iv) *Escherichia coli* growth rate model of Ross *et al.* (2003)
v) Updated probability of growth model for *L. monocytogenes strains* Scott A and L5 developed at University of Tasmania based on the original models of Tienungoon *et al.* (2000; *see* Appendix 10)
vi) *Escherichia coli* probability of growth model of Presser *et al*. (1998)

All of the above models include terms that are used to derive the undissociated lactic acid concentration as a predictor variable, in addition to temperature, pH and water activity or aqueous salt concentration.

The Augustin *et al*. (2005) model is specific for *L. monocytogenes.* Its intended application includes dairy products including those containing lactic acid. The effect of lactic acid is not explicitly modelled and, instead, the user selects a variant of the model that is appropriate to foods containing lactic acid. The predictions of probability of growth from that model require, however, only temperature, pH and water activity data. The Augustin *et al*. (2005) model '#8bis', was also

evaluated for every time point of every challenge trial (*see data file*: All Challenge Trial Data). Despite that growth stasis and inactivation of *Listeria* spp. was evident at some point in all cheeses, only ~ 20 of the > 400 observations (20 trials - 5 cheeses x 2 species x 2 milk types [raw or pasteurised] with an average of 20 sets of measurements each) produced probability of growth estimates < 0.50, and most predict probability of growth greater than 0.9, even when the data showed pathogen inactivation. In only two cases was the probability of growth predicted by the Augustin *et al.* (2005) model "#8bis' less than 0.1, the level considered as a reliable prediction of 'growth not possible'. For three of the four cheddar trials, the model predicted 'no growth' after the fermentation step but, for the fourth trial, generated an apparently erratic pattern of no-growth predictions despite that, overall, that trial appeared similar to the other three. The Augustin *et al.* (2005) model did not predict the transient inactivation observed in the double-cream Brie challenge trials.

The poor performance of the Augustin *et al.* (2005) model may be due to difficulties in determining the water activity of cheeses, and the assumption that salt and water content would be enough to establish water activity of cheeses because salt is not the only compound that contributes to the water activity. In the challenge trials performed in this study, water activity was directly measured in each cheese, at each sample time, using a dew point instrument. Accordingly, the water activity values used were accurate and not subject to estimation assumptions, but the model still did not produce reliable predictions. Accordingly, there may be other reasons for the inadequate performance of the models. Given our experience that the Augustin *et al.* (2005) model offers relatively poor ability to discriminate growth-permissive from growth-preventing conditions it was not considered further in this study.

The absence of undissociated lactic acid as a predictor variable is a possible explanation for the apparently poor performance of the Augustin *et al.* (2005) '#8bis' model. From examination of the data in Appendix 9, it can be seen that undissociated lactic acid concentrations alone can account for the observed growth inhibition in many cases, in addition to hurdles imposed by reduced pH or water activity. As described below, in this study, models that included terms for undissociated lactic acid produced a very high proportion of correct predictions of growth/no growth both for *Listeria* spp. and *E. coli* strains.

The Schvartzman *et al.* (2010) might also be considered to be relevant but its applicability is limited due to lack of consideration of dynamic changes, limited range of relevant predictor variables, and because it considers one temperature (·30°C) only. Accordingly, the Schvartzman et al. (2010) model was not further considered in these studies.

Mejlholm and Dalgaard (2009) presented a more comprehensive version of their earlier model (Mejlholm and Dalgaard, 2007) that includes terms for the influence on growth of other organic acids in addition to lactic and acetic acids. In their analysis of growth/no growth models relevant to cheese. FSANZ (2014), in an analysis of available models, discounted consideration of the Mejlholm and Dalgaard (2009) model because "lactic acid and acetic acid concentration data was not available" from the collation of 34 challenge trials datasets in the published literature that they used to assess available models. This was unfortunate because, as will be demonstrated using the challenge trial

data generated in the current study, the Mejlholm and Dalgaard (2007) model produces a very high proportion of correct predictions of growth/no growth of *Listeria* spp. for a variety of cheeses. To facilitate the analysis, an editable version of the Mejlholm and Dalgaard (2007) model was obtained from Dr. Paw Dalgaard, Danish Technical University, which enabled predictions to be made by extrapolation beyond the nominated prediction ranges of the model that is available on-line (i.e., *see* http:// sssp.dtuaqua.dk/). This was necessary for predictions during the acidification, curd formation and moulding stages of the cheese making, for which temperatures were typically in the range 27 – 38°C. To be rigorous, the Mejlholm and Dalgaard (2007) model is suggested to be limited for use with temperatures up to 15°C only, as predictions beyond this range are extrapolations beyond the range of the data used to generate the models. However, numerous other published studies using 'square-root-type' models have shown that extrapolation up to ~35°C would yield reasonable predictions . Similarly, the editable version of the model allowed % aqueous phase NaCl values <1.5% to be used to make predictions.

For predictions using the modified-UTas models for *L. monocytogenes* probability of growth, an upper default temperature of 25°C was used because the model generates 'imaginary' numbers at for temperatures above 30°C. The consequence of the assumed default value would be expected to lead to higher predicted probabilities of growth because 25°C is closer to the centre of the normal physiological range of *L. monocytogenes*. Appendix 9 provides additional details about assumptions made about "missing" data to enable predictions to be made, and why those assumptions were necessary.

Absolute growth limits for both *L. monocytogenes* and *E. coli* have been described by many researchers, but are not completely consistent. Differences are due in part to growth media, and also differences between strains (Begot *et al*., 1997; Salter *et al.*, 2000; Shabala *et al.,* 2008). Nonetheless, representative values for absolute limits to growth of *L. monocytogenes* and *E. coli* are shown in Table 5.8, below. Temperature, pH, water activity and undissociated lactic acid limits for *E. coli* are derived from Presser *et al.* (1998) and Ross *et al.* (2003), while temperature, pH and water activity limits for *L. monocytogenes* growth were collated from the published literature and presented by Ross *et al.* (2001). The undissociated lactic acid limit is an approximation derived from Tienungoon *et al.* (2000) and Coroller (2005; *cited in* Schvartzman *et al*., 2011).

Table 5.8 Representative *absolute* limits to growth of *E. coli* and *L. monocytogenes* to environmental factors of relevance in cheeses

	lower limit for growth				upper limit for growth			
	Temperature (°C)	pH	a_w	undissociated lactic acid (mM)	temperature (°C)	pH	a_w	undissociated lactic acid (mM)
E. coli	7.5	3.9	0.95	n.a.	50	10	n.a.	10
L. monocytogenes	-1	4.3	0.92	n.a.	46	10	n.a.	5

From these absolute growth-preventing values, and the data describing the physico-chemical characteristics of the various cheeses it is possible to identify non-growth conditions from the data presented in the tables in Appendix 9. Although Codex (CAC, 2007) consider that foods in which pH is <4.4, in which a_w <0.92, or foods with both pH < 5 and a_w <0.94 can be considered to preclude growth of *L. monocytogenes*, there are few examples of cheeses (see Section 1), that satisfy these criteria. Nonetheless, it has been reported that many cheeses that do not satisfy these criteria nonetheless do *not* support the growth of *L. monocytogenes*, indicating that other physico-chemical factors are involved, with organic acids being the most obvious candidates in fermented foods. Examination of the data in Appendix 9 reinforces these observations.

The idea that growth-inhibiting factors (or sub-optimal conditions) interact to reduce the growth permissive range for any single physico-chemical factor is well established in the food microbiology literature. It is described as the "Hurdle Effect' and exploited in "Hurdle Technology" or 'combination preservation' or 'multiple' barrier technologies (Gorris, 2001). In addition to the data presented in Appendix 9, growth permissive, and non-permissive conditions for both *E. coli* and *Listeria* spp. can also be discerned from Figures 5.1 to 5.5.

The inclusion of predictions of growth/no growth based only on individual absolute limits to growth enables the significance of the Hurdle Effect in cheeses to be evaluated because the growth/no growth models would be expected to encompass the consequences of the Hurdle Effect, while they are ignored when probability of growth is estimated by the absolute limits individually. As such, more predictions of growth would be expected from the latter approach than the dedicated growth/no growth model. As can be seen from Appendix 9, this was observed.

In total, there were 260 observations (130 each for *Listeria* spp., and 130 for *E. coli* strains) for which sufficient data, including lactic acid data and use of 'default' values, were available to make growth/no growth predictions. Some of those predictions relate to situations in which growth is highly likely (i.e., before pH began to fall after addition of the starter cultures) or highly unlikely, e.g., conditions are stable and no further change in growth potential would be observed. Accordingly, for all but Brie cheese, only three observations before (from the graphs) growth ceased were used for predictions from simple absolute limits model. The double-cream Brie challenge trials are an exception because growth subsequently resumed, as noted earlier. Accordingly, for the Brie challenge trials only, predictions were made for all observations in the challenge trials.

Among the 260 observations of growth/no-growth (judged from the figures presented in Sections 4 and 5) and physico-chemical parameters, only 24 predictions from the simple model differed from the more complex growth/no growth models. Of the 24 differences, 20 related to *E. coli,* while only four related to *L. monocytogenes.* In all cases differences were because the simple model predicted 'growth possible' while the complex models predicted 'no growth'.

This suggests that the more complex models, particularly for *E. coli*, provide more reliable growth/no growth predictions but that it may be less important for the predictions of *L. monocytogenes* probability of growth.

As noted, the data are coarse, with relatively rapid changes occurring during the later stages of acidification, curd formation and moulding. In other words, for most of the data there is little discrimination of model performance afforded by the data because most of the data are far to the growth side, or to non-growth side of the growth/no growth interface. To increase the rigour of the evaluation, for each challenge trial only the three observations before the observed cessation of growth, and three after the observed cessation of growth of the challenge organism were included in the evaluation. For the double-cream Brie challenge trials, growth of the challenge strains resumed during maturation. Accordingly, for the Brie comparison, the three observations prior to growth cessation, the three during growth cessation (in three out of four challenge trials) and the three after resumption of growth (as judged from the Figures in Section 4 and 5) were used. This resulted in 90 observations for both *Listeria* spp., and 90 for *E. coli*: 24 observations in Cheddar cheese, 24 observations in Gouda, 24 observations in Feta and 18 observations in double-cream Brie. Table 5.9, below, summarises the number of discordant predictions (*cf.* observed responses) for each type of cheese and each model.

Table 5.9 Summary of growth/no growth model performance

	Number (%) of discordant [a] predictions			
	Listeria monocytogenes		*E. coli :*	Number of observations
Model:	Mejlholm and Dalgaard (2007)	"UTas"	Presser *et al.* (1998)	
Cheese type				
Cheddar	3 *(12.5)*	3 *(12.5)*	3 *(12.5)*	24
Gouda	2 *(8)*	1 *(4)*	2 *(8)*	24
Feta	7 *(29)*	10 *(42)*	4 *(17)*	24
Brie	0	0	0	18
TOTAL	12 *(13)*	14 (16)	9 (10)	

[a] *n.b.* All erroneous predicted were 'fail safe', i.e., growth predicted as possible when it was not observed.

These results compare well to reported growth/no growth model performance, especially given that the models are not designed specifically for cheese, nor were the test data used to generate the models. Furthermore, the data were specifically chosen to be close to the growth/no growth boundary and it was noted that, for the Cheddar and Gouda data, all but two of the discordant predictions were for sampling times adjacent to the growth/no growth boundary, as judged from the relevant plots of the data. For Feta, however, the results were more erratic and discordant results did not necessarily arise from observations 'adjacent' to the observed growth/no growth boundary.

This evaluation of model performance is less than ideal because the values used for model predictions do not always relate exactly to the time of viable count determination, particularly the lactic acid concentration values which were assessed less frequently due to the complexity and financial and time cost of the assay. As noted, some values used related to data collected hours before the corresponding viable count and pH values were measured, so that there is expected inaccuracy in many of the lactic acid concentration values. This is particularly important because of the effect of acidification, together with the evolution of lactic acid, and the rapidly changing and increasingly hostile environment during this process. pH and undissociated lactic acid concentration might be expected to change exponentially over time due to the exponential growth of lactic acid bacteria starter cultures (*i.e.*, which produce lactic acid from catabolism of lactose, which in turns reduce pH which in turn increases undissociated lactic acid concentration). Given these rapidly changing conditions, more accurate data might be expected to generate fewer discordant predictions from the models. A possible improvement would be to estimate intermediate values from measurements before and after the relevant interval for which specific lactic acid concentration data is missing but, because of the rapidly, and exponentially changing pH and lactic acid concentration, this was not attempted at this time.

Nonetheless, given the methodological and data limitations, the performance of the models is, *at least*, encouraging and suggests that such models can be used reliably to identify conditions in cheeses that preclude growth of specific pathogens.

5.4 Conclusions and Interpretations

The aims of this work have been to:

iv) assess the relative safety of analogous cheeses made from raw or pasteurised milk
v) increase understanding of the ecology and fate of microbial pathogens in cheeses and also
vi) evaluate whether predictive models can provide sufficiently reliable predictions to support risk management decisions about specific cheeses and cheese making processes based on raw or pasteurised milk, and
vii) consider how the results and analyses can contribute to science-based management of the microbiological safety of cheeses, including those made from raw milk.

While the data presented here are representative of cheese making for the various style of cheese used in the challenge trials, there are many other variables. For example, in this work, only two types of starter culture were used, and it is also known that maturation times and maturation conditions can vary among different processes for the same nominal cheese type. None of the cheeses made involved a lethal heat treatment and, accordingly, we have not sought to evaluate models for thermal inactivation of pathogens potentially present in cheese. Nonetheless, the characteristics of the cheeses produced in this project are generally consistent with the summary of characteristics presented in FSANZ (2014) for different styles of cheese. A possible exception is the Feta cheese, which did not achieve pH as low as intended.

5.4.1 Evaluation of Models

While the data were imperfect, for reasons discussed in Section 5.3.10, the performance of the growth/no growth models evaluated was at least encouraging. Use of a simple model based on the absolute limits of growth for each organism showed that the Hurdle Effect was evident in providing microbiological stability of cheeses, with the complex growth/no growth models (i.e., that implicitly include the consequences of the Hurdle Effect) producing more 'correct' predictions of 'growth' or 'no growth'. ***Most importantly, there were no predictions of 'no growth' if growth was, in fact, observed.*** All erroneous predicted were 'fail safe', i.e., growth predicted as possible when it was not observed.

Models for growth rate also provided reasonable predictions of growth rate during the acidification stages in most cases though several unexplainable deviations between growth rate and predictions were evident. Also, growth of challenge organisms seemed to cease sooner in the process than was predicted from the available physicochemical data over time.

For evaluation of either type of model more detailed, *i.e.*, more frequent sampling times during acidification, and more complete sets of measurements, e.g., pH, a_w, lactic acid, acetic acid are required to evaluate the models. This is because of the number of physico-chemical factors enabling or preventing the growth of the challenge organisms and because those factors are changing relatively rapidly and significantly in magnitude throughout the early phases of the trial. Nonetheless, the performance of the models was encouraging.

Investigation of other factors that affect microbial growth potential, and growth rate should also be considered. For example, it was noted that estimated growth of challenge strains was slower in the only cheese made with goat's milk and pathogen growth was usually slightly more prolific in cheeses made from pasteurised milk. Reliable explanations for these observations are not available.

5.4.3 Risk Management Approaches

To develop food safety risk management approaches for making raw milk cheeses, it must first be resolved whether the risk (of pathogen growth or survival) is greater in cheeses made from raw or pasteurised milk. From the results presented here, there is no significant difference. While pathogen growth was slightly greater in most pasteurised milk cheeses than equivalent raw milk cheeses, pathogen inactivation was also faster in cheeses made from pasteurised milk. Notably, however, pathogen levels initially present in the milk used to make cheese increased in all cheeses during cheeses formation, typically by 2 $\log_{10}$CFU. For E. coli challenge studies, the increase was higher ($\bar{x}$ =2.22 $\log_{10}$CFU, SD = 0.46, n= 20), while average increase for *Listeria* spp. was slightly less ($\bar{x}$ =1.55 $\log_{10}$CFU, SD = 0.39, n = 19).

Accordingly, milk must have initial pathogen levels that are not a threat to human health, and also be matured for long enough, and under growth-preventing conditions, to eliminate 2.2 $\log_{10}$ *E. coli* and 1.55 $\log_{10}$ *L. monocytogene*s if the levels initially present in milk are considered safe.

As demonstrated in Section 4, the effect of temperatures on the inactivation rate of both sets of challenge studies was highly reproducible, even though absolute inactivation rates were variable, Nonetheless, as a first approximation it is possible to derive an empirical, conservative, inactivation model for both pathogens from the data presented in Figure 4.5d. The model, based on encompassing the data for slowest inactivation rates is:

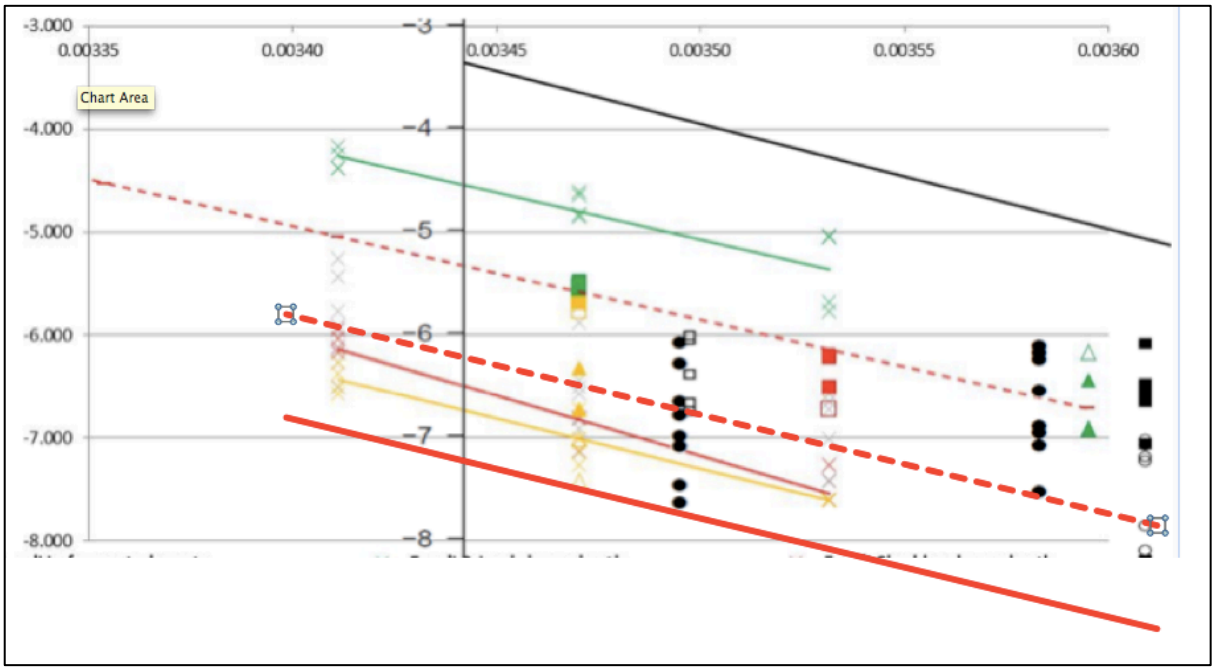
ln(inactivation rate) = -9500 x 1/Temperature (K) + 25.5

Alternatively, a model that approximates average inactivation rates is:

ln(inactivation rate) = -9500 x 1/Temperature (K) + 26.5

A comparison of these two entirely empirical models to the available data from this study is depicted in Figure 5.9.

From these empirical equations, predicted times required to reduce *E. coli* and *Listeria* spp. to their starting levels in milk can be estimated to give an indication of the time required for maturation at different temperatures, based on the idea of 'no net increase' before the product is able to be sold. These predictions are shown in Table 5.10, for both conservative assumptions and for average expected inactivation rates and pathogen increases during cheese-making. It is noted that the former USFDA "60 day rule" is, on average, at least 'in the right ball park' for typical maturation temperatures for many cheeses, e.g., around 15°C, plus or minus a few degrees. However, the conservative values are approximately 2.7 times longer. The USFDA rule was based largely on experience and so it is perhaps unsurprising that it was formerly used with reasonable success. However, the increasing incidence of failure of the 60-day rule may result from more cheeses being manufactured so that the less likely scenarios (worst cases) are becoming frequent enough to be noticed as outbreaks.



Figures 5.9 Graphic comparing the two empirical models for 'safe' pathogen inactivation rates in all cheeses. The solid red line is the conservative model, while the dashed red lime depicts the model intended to indicate average inactivation rates. Details of the data were given in Figure 4.5d.

Table 5.10 Estimated times (days) for pathogen reduction to starting levels in milk based on data in Figure 4.5d and average increases in pathogen during acidification and curd formation

Temperature (°C)	days required for 2.2 log10 inactivation		1.5 log10 inactivation	
	average	conservative	average	conservative
5	193	525	132	358
10	106	287	72	196
12	84	227	57	155
14	66	180	45	123
15	59	160	40	109
16	53	143	36	98
18	42	114	29	78
20	34	91	23	62

From the data in the above Table, it is possible to evaluate, at least at a first approximation, times required for maturation of cheeses if the cheese is produced with levels of pathogens that would not be expected to lead to public health. To put those estimates into perspective, typical maturation times and temperatures for various styles of cheeses are:

Cheddar – 90 to 750 days at 10°C

Gouda – 60 to 90 days (but up to 2 years) at 15°C

Feta – 30 to 60 days at 12- 15°C, and refrigerated storage on brine for many months

Brie – 14 to 30 days at 12°C

Wensleydale - 30 – 90 days at 15°C

It would appear that these time-temperature combinations are probably adequate for the average situation, but would be expected to occasionally fail to reduce pathogen levels in the final curd back to the levels in the milk. It should be remembered that the above are very crude first estimates of the needs of some generic cheeses style. Important, and reproducible, differences in pathogen inactivation rates were observed between Feta and other cheeses, so that recognition of the specific properties of the cheese may be appropriate to reduce, or lengthen, the conditions of maturation

5.4.4. Potential and tolerable levels of pathogens in raw milk used for cheese making

For *E. coli*, the expectations of tolerable levels in cheese differ in different jurisdictions. Much higher loads of *E. coli* are tolerated in cheese in Europe, for example, than in Australia. On the other hand, for *L. monocytogenes* levels of up to 100 $cfu.g^{-1}$ are now considered by The Codex Alimentarius Commission and the European Food Safety Authority to be tolerable in foods that do not support growth of *Listeria*.

While oft-cited anecdotal reports suggest that 'as few as 100 cells of enterohaemorrhagic *E. coli* could cause infection, illness and death' FAO/WHO (2011), however, conducted an analysis of currently available dose response models for EHECs, including Strachan *et al.* (2005) and Cassin *et al*. (1998). The Cassin *et al.* (1998) model was shown to provide a good representation of the average predictions of those models, while Strachan *et al.* (2005) presented evidence of more extreme cases. From the FAO/WHO (2011) model, the ID_{50} for EHEC infections can be characterized as having a mean of ~2500 cells (ID_{50} = 3.4 $\log_{10}$cells), but with a standard deviation of 0.9 $\log_{10}$cells (i.e., giving a 95% confidence interval of 40 to 155,000 cells).

Tolerable levels of pathogens in foods, however, must be much lower than the ID_{50} level. For example, for *L. monocytogenes*, recent studies by Smith and colleagues in USA using pregnant monkeys and guinea pigs[2] as experimental models for oral infectious doses (Smith *et al*., 2003, 2008; Williams *et al*., 2007; Williams *et al.*, 2009) suggest an ID_{50} for pregnant women is ~ 10^8 cells. To further place that risk into context, pregnant women are estimated to be 10 – 100 times more

[2] Both animals carry the E-cadherin gene that encodes a protein required for *Listeria* attachment and initiation of infection.

susceptible to listeriosis than people with no conditions that would pre-dispose them to listeria infections (Goulet *et al.*, 2012). Note that this estimate of the ID_{50}, based on more recent data, is *considerably* lower than that estimated by FAO/WHO (2004).

If a total dose of ~10000 cells, in a 100 g portion of food is now implicitly considered tolerable, this implies a 'safety margin' of 1,000 to 10,000-fold below the ID_{50} as being 'reasonable'. If the same strategy were applied to pathogenic E. coli, tolerable levels in foods would be in the range a few cells per kilogram of food, or per litre of raw milk. Ross (2011) reviewed the literature on potential levels of pathogens in raw milk and founds that levels of 10^4 to 10^5.ml^{-1} could be expected in some circumstances but that those circumstances would not always be easily recognisable. From the above results and discussion these potential levels are much higher than normal cheese making processes could be expected to reduce to tolerable levels and is, of course, one of the reasons that pasteurisation of milk for cheese-making has been mandated in many jurisdictions.

5.4.5 Effectiveness of testing

The above analysis leads to consideration of whether, given pathogen inactivation expectations for different styles of cheese/maturation conditions, and the implicit maximum initial pathogen loads required for safe finished cheeses, how those initial levels might be assured. As noted, heavily contaminated milk is not readily apparent to the sense, usually requiring microbiological analysis. Also, infections in udders that lead could to high pathogen loads in milk are not always evident and only a gram amounts of faeces from a cow shedding high levels of EHECs would be sufficient to contaminate milk to a level unacceptable for raw cheese making.

Using existing methods detection of unacceptable levels of *Listeria monocytogenes* in bulk milk could readily be achieved with a simple direct plate test. In this test 0.1 ml of milk is spread on to the surface of an appropriate selective agar surface. If ten or more colonies are detected it would infer that the concentration in the milk exceeds 100.ml^{-1}. Natural inhomogeneity in the distribution of cells in milk could, however, mean that a single test sometimes will contain more than the average amount in the entire batch. Accordingly multiple samples should be tested and an average count obtained. Software tools, such as those developed and made freely available by the International Commission for Microbiological Specifications for Foods (http://www.icmsf.org), can assist in design of sampling plans that achieve the needed amount of confidence that false positive will be infrequent. Combined with use of models to ensure that the final product does not support growth, and assurance that the rate of acidification is within the expected range (i.e., to limit growth of the pathogen during the initial acidification phases), testing could provide protection against highly contaminated raw milk being used. However, the results of testing would not be available for one to two days meaning milk would have to be stored, or that a batch might have to be discarded if test results were positive. In addition, the same standards for in-plant hygiene as apply to the manufacture of cheese from pasteurised milk, would need to be assured.

Testing for *E. coli* is likely to be more problematic. The problem of testing for low numbers of cells in a batch of food is that a single sample volume will only have a low probability of a cell being present. For example, in the discussion above, a level of ≤1 enterohaemorrhagic *E. coli* per litre of milk may

be required to ensure the safety of the final product. Testing for this low level is more labour- and cost-intensive. For example, if 25 mL units of milk were sampled, it would be expected that on average, less than one in 40 such samples of a batch of milk that *complies* with the required level would 'test positive'. Using the binomial distribution, it can be shown that to be 95% confident that the contamination rate is less than one in 40 (and that the contamination level in the milk is less than one CFU per litre), would require testing of 119 samples of 25 mL from each batch of milk used for cheese making, requiring nearly three litres of milk. NZMPI (2014b) explored this topic in greater detail. An alternative might include testing for 'generic' *E. coli* but the relationship between 'generic' *E. coli* is uncertain and, probably, sporadic.

Moreover, there are other low infectious dose pathogens that could be present in raw milk, such as Salmonella. FAO/WHO (2002) estimated ID_{50} in the range of hundreds to thousands of cells, but a more intense and recent estimate by Teunis *et al.* (2010) is in the range of tens to hundreds of cells. It would seem that such low dose pathogens present an intractable problem for the safe manufacture of raw milk cheeses unless those cheeses have long ripening times at relatively high temperature so that the maturation can be considered a reliable 'critical control point'. This could significantly increase the cost of testing.

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6 Inactivation kinetics of *E. coli* and *Listeria* spp. in milk-based broths as a function of undissociated lactic acid concentration and pH

6.1 Introduction

Despite one of the original hypotheses implicit in this project, it became evident from our earlier experiments (*see* Section 2) that inactivation responses of bacteria might not only be affected by temperature, *but also* by pH, organic acid content, water activity etc. Specifically, brined cheeses were found to have much lower pH and higher undissociated lactic acid concentrations than other types of cheeses studied and inactivation of both *Listeria* spp. and *E. coli* in the broths that emulated brined cheeses was different to that in other cheese-like broths. Their inactivation appeared to commence almost immediately after inoculation into the simulated brined-cheese broth, and the absolute rates of inactivation of bacteria in brined-cheese-like broth were faster than those in other cheese-like broths. To further investigate the bases of these observed differences, experiments were undertaken to attempt to differentiate the effects of pH and undissociated lactic acid levels on inactivation responses of *Listeria* spp. and *E. coli* because these factors were most different in brined cheeses compared to other types of cheese (*see* Table 1.2).

6.2 Approach

A series of broths based on milk medium (1% full cream milk powder, 0.5% peptone and 0.3% yeast extract) were prepared with the same water activity (a_w 0.945, representative of a broad range of cheese and a growth-preventing factor alone for *E. coli*) but different levels of pH and lactic acid. The conditions tested were the combinations of the highest or lowest values for pH and lactic acid concentration based on the chemical properties of all cheeses previously characterized in our earlier study (*see* Section 1). Table 4.1 describes the parameters for each condition used in this study. All broths were inoculated with cultures of *Listeria* spp. (*L. monocytogenes* or *L. innocua*) or *E. coli* (R31 or M23) and incubated at 15 and 25°C. Pathogen survival was assessed as a function of time.

Table 6.1. Chemical conditions of nutrient-rich ("milk-based medium") broths used in this study[a].

Number	pH	L-lactic acid (g/100g)	Undissociated lactic acid (mM)
1	7.55	0.01	0.00
2	7.55	2.53	0.06
3	4.14	0.01	0.38
4	4.14	2.53	96.75

a. All conditions were tested at a_w 0.945.

On the basis of the chemical parameters for all test conditions (including the a_w hurdle), from prdictive microbiology models (Presser *et al*., 1998; Meijholm and Dalgaard, 2007) neither target bacteria would be expected to grow. This is because the conditions are beyond the limits for their growth. Furthermore, it was expected that the condition that has the lowest pH and highest undissociated lactic acid concentration (i.e., pH 4.14, undissociated lactic acid 96.75 mM) is the most hostile for the bacteria, whereas the condition that has highest pH and lowest lactic acid concentration (i.e., pH 7.55, no undissociated lactic acid) is the least hostile.

6.3 Results and Disussion

In all casses, a biphasic inactivation response was observed for both *Listeria* spp. and *E. coli* (Figures 6.1 and 6.2). *Listeria* spp. entered a lag phase for a period of time before inactivation commenced. For *E. coli*, an initial fast phase of inactivation was observed followed by a much longer second, slower, phase of inactivation. These patterns of inactivation are consistent with the obserivations in our previous trials on inactivation kinetics of bacteria in cheeses and simulated cheese broths (*see* Sections 2 and 3). Furthermore, it was evident that the biphasic inactivation response was more pronounced in some conditions than others and more pronounced for *E. coli* inactivation. Of particular note was that the biphasic response was less obvious for both *Listeria* spp. and *E. coli* under the condition at pH 4.14 and undissociated lactic acid concentration of 96.75 mM. This may be due to the fact that this condition is the most hostile and, thus, bacterial inactivation occured at much faster rate than other conditions tested. Accordingly, the same explanation may account for earlier observation in which biphasic inactivation was not apparent in the inactivation kinetics of *Listeria* spp. and *E. coli* in the broths that emulated brined cheeses.

Inactivation rates for each species and strain under each condition were calculated by linear regression analysis. In the case of *Listeria* spp., rates in the second, faster, phase of inactivation were reported. In the case of *E. coli*, rates in the second, slower, phase of inactivation were estimated and collated. Inactivation rate data are summarised in Figures 6.3 and detailed in Appendix 5. Arrhenius plots were generated and used to describe inactivation rates as a function of temperature for each strain of each species/genus, as shown in Figures 6.4. The average rate of inactivation of *E. coli* in fermented sausages and related broths, as determined by McQuestin *et al.* (2009) was also included for comparison.

Figures 6.3a, b describe the inactivation rates of both *Listeria* spp. and *E. coli* under conditions of different levels of pH and undissociated lactic acid at each temperature tested. It was evident in all cases that inactivation at pH 4.14 and undissociated lactic acid concentration of 0.38 mM was slower than that observed at the same pH but higher level of undissociated lactic acid (i.e., 96.75 mM). Inactivation at the low concentration of undissociated lactic acid was less than half as fast as that at the high undissociated lactic acid concentration. However, rates of inactivation at high pH (i.e., pH 7.55) were found to be very similar between the conditions without undissociated lactic acid and with very low level of undissociated lactic acid concentration (i.e., 0.06 mM), although the low level

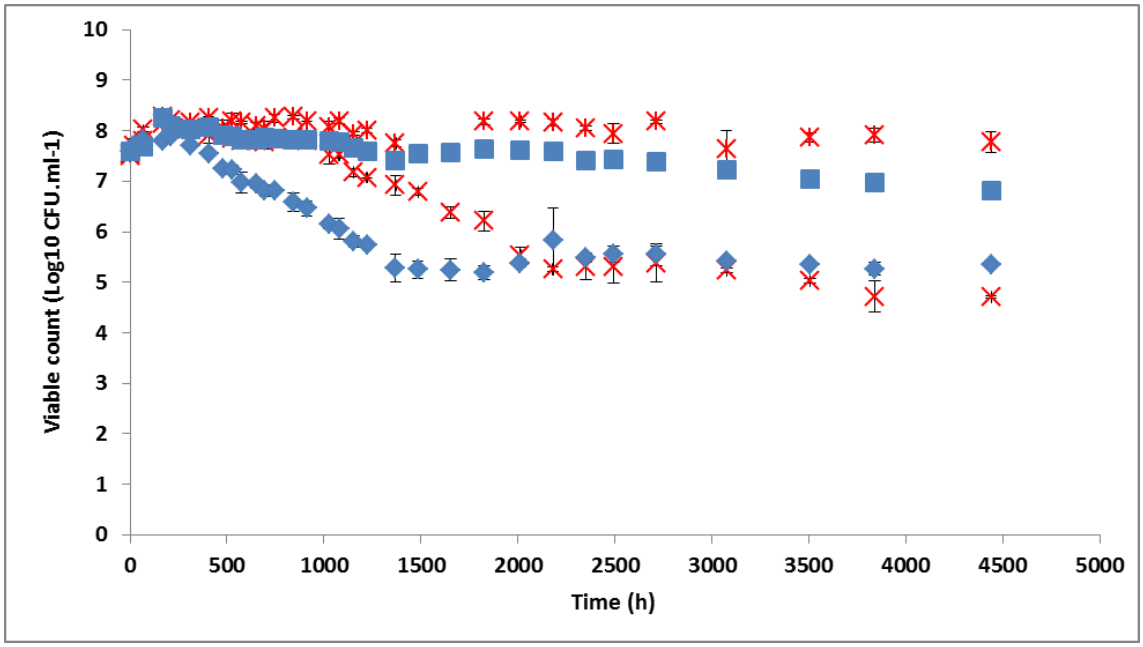

Figure 6.1a. Inactivation data for *Listeria* spp. in milk-based broth at a_w 0.945, pH 7.55, no undissociated lactic acid. *L. monocytogenes* Scott A incubated at 15°C (✳) and 25°C (×); and *L. innocua* at 15°C (■) and 25°C (◆).

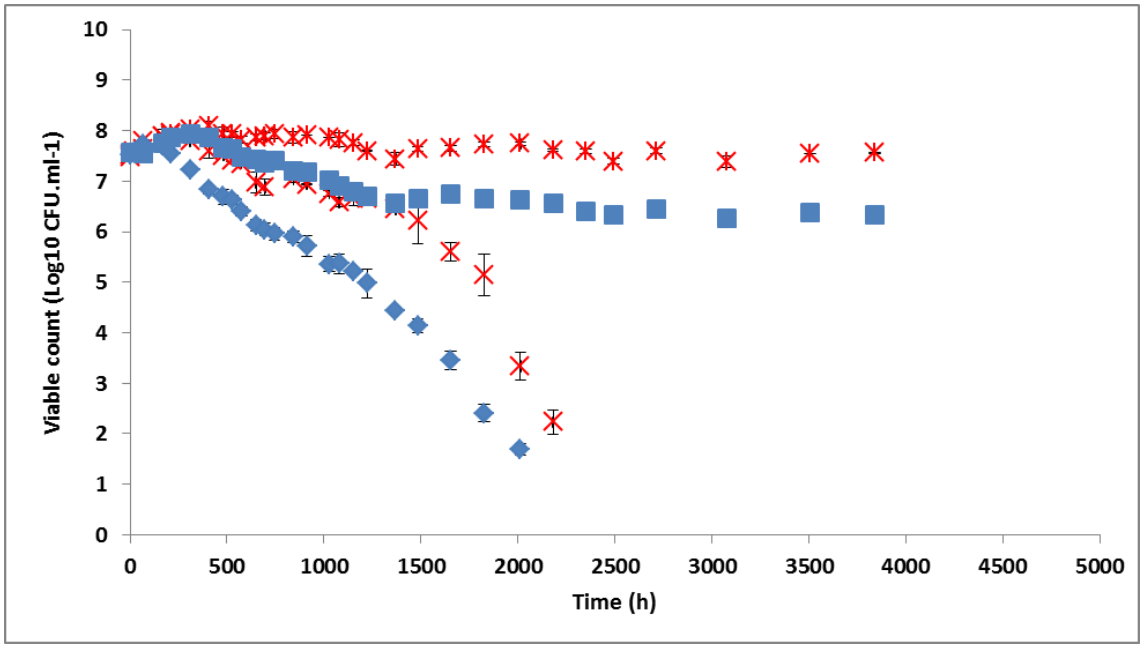

Figure 6.1b. Inactivation data for *Listeria* spp. in milk-based broth at a_w 0.945, pH 7.55, undissociated lactic acid 0.06 mM. *L. monocytogenes* Scott A incubated at 15°C (✳) and 25°C (×); and *L. innocua* at 15°C (■) and 25°C (◆).

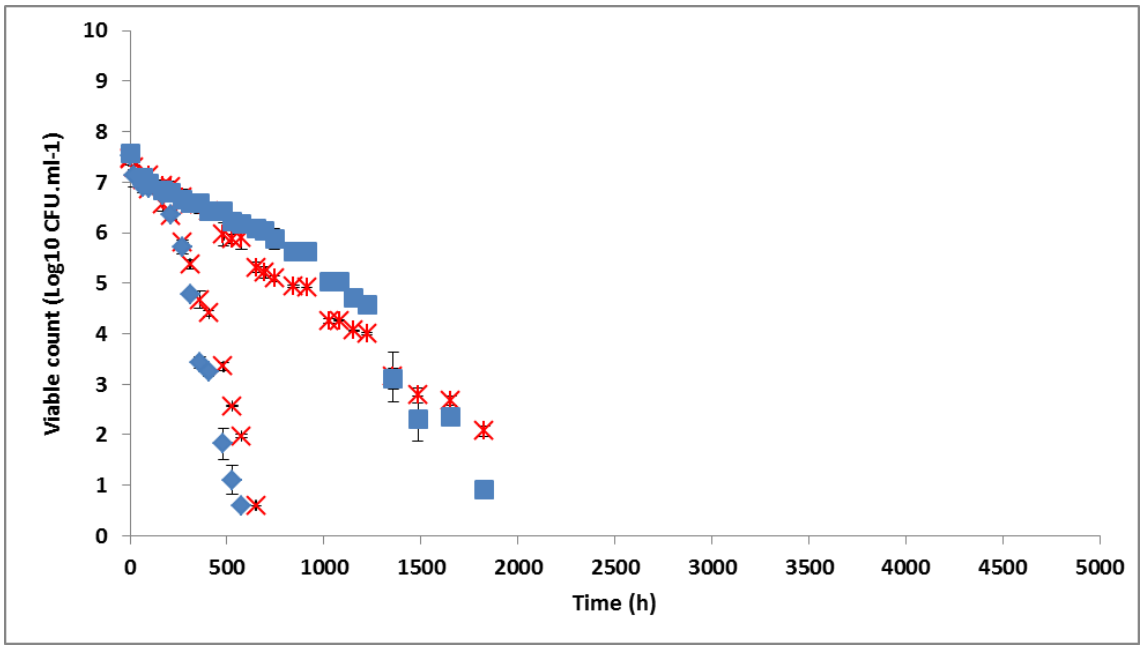

Figure 6.1c Inactivation data for *Listeria* spp. in milk-based broth at a_w 0.945, pH 4.14, undissociated lactic acid 0.38 mM. *L. monocytogenes* Scott A incubated at 15°C (✳) and 25°C (×); and *L. innocua* at 15°C (■) and 25°C (◆).

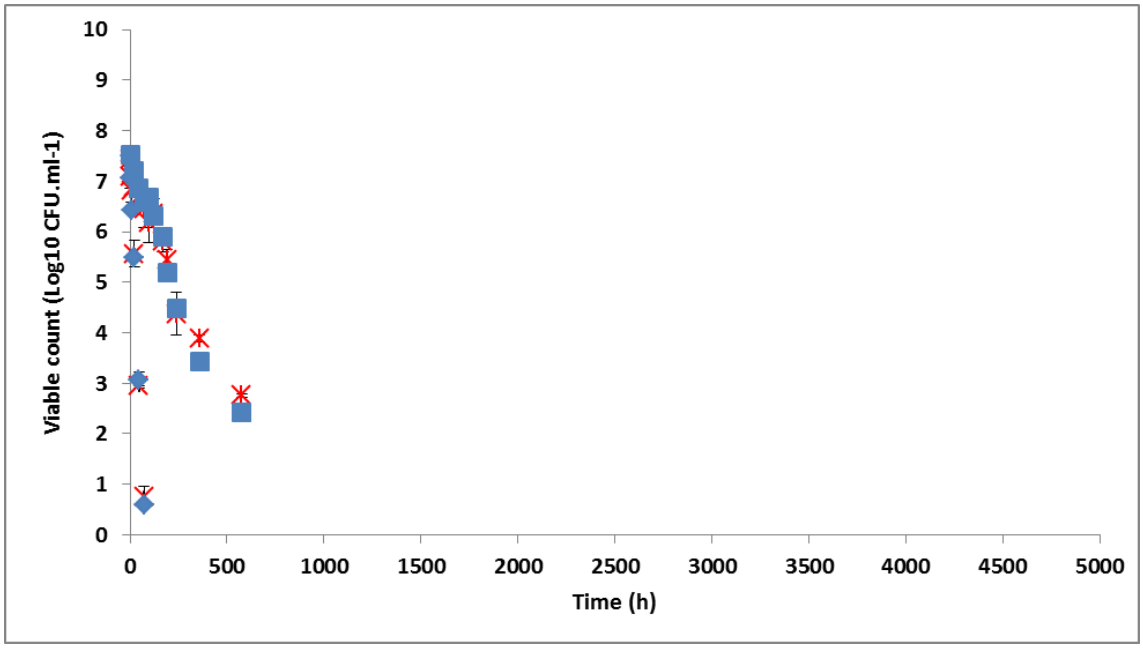

Figure 6.1d Inactivation data for *Listeria* spp. in milk-based broth at a_w 0.945, pH 4.14, undissociated lactic acid 96.75 mM. *L. monocytogenes* Scott A incubated at 15°C (✳) and 25°C (×); and *L. innocua* at 15°C (■) and 25°C (◆).

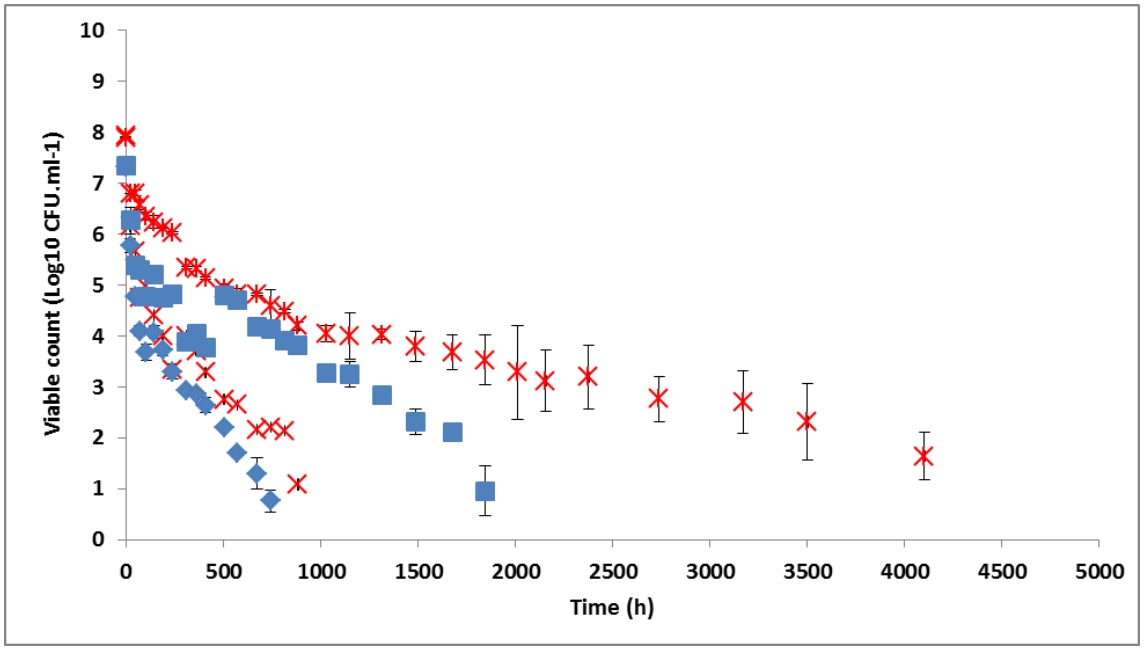

Figure 6.2a **Inactivation data for *E. coli* in milk-based broth at a_w 0.945, pH 7.55, no undissociated lactic acid.** *E. coli* R31 incubated at 15°C (✳) and 25°C (×); and *E. coli* M23 at 15°C (■) and 25°C (◆).

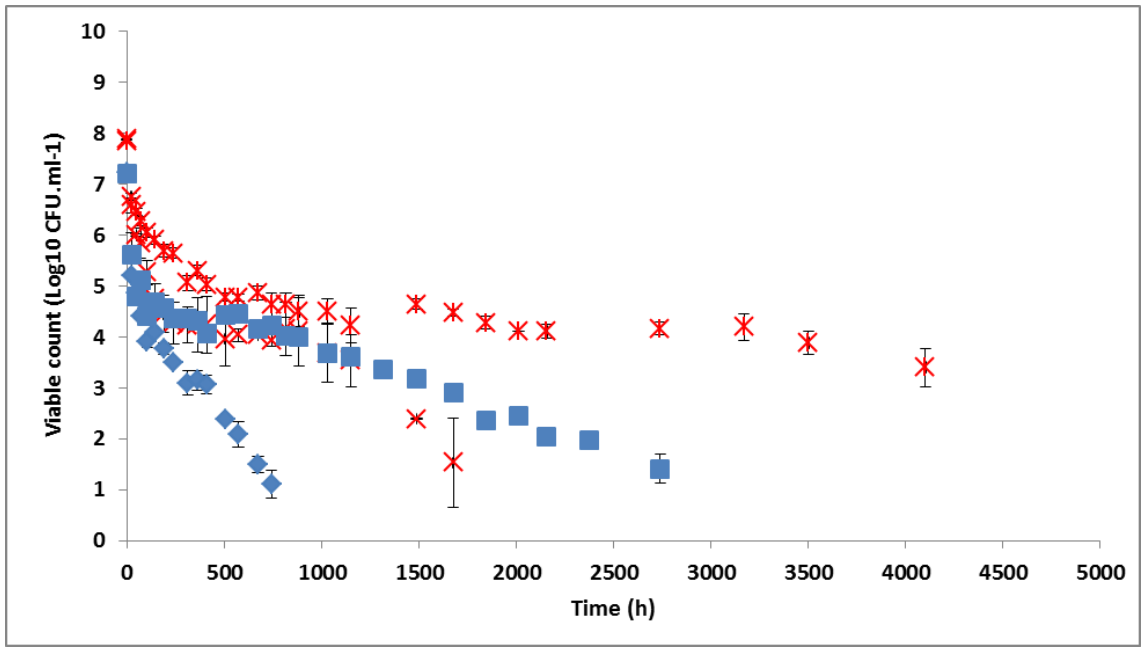

Figure 6.2b **Inactivation data for *E. coli* in milk-based broth at a_w 0.945, pH 7.55, undissociated lactic acid 0.06 mM.** *E. coli* R31 incubated at 15°C (✳) and 25°C (×); and *E. coli* M23 at 15°C (■) and 25°C (◆).

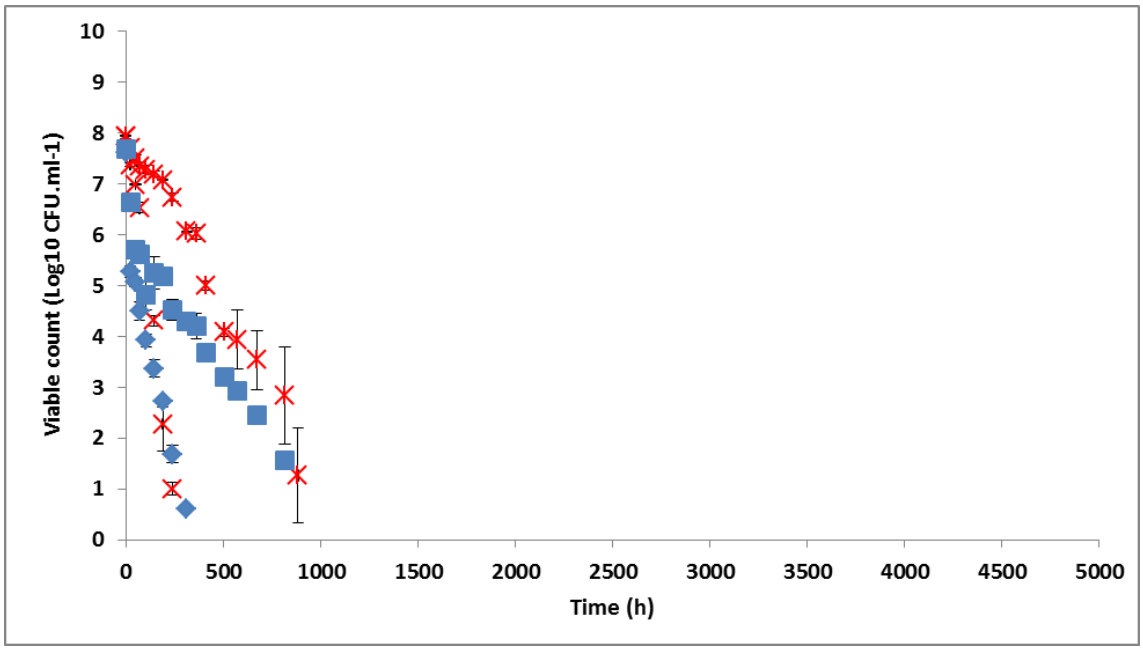

Figure 6.2c Inactivation data for *E. coli* in milk-based broth at a_w 0.945, pH 4.14, undissociated lactic acid 0.38 mM. *E. coli* R31 incubated at 15°C (✳) and 25°C (×); and *E. coli* M23 at 15°C (■) and 25°C (◆).

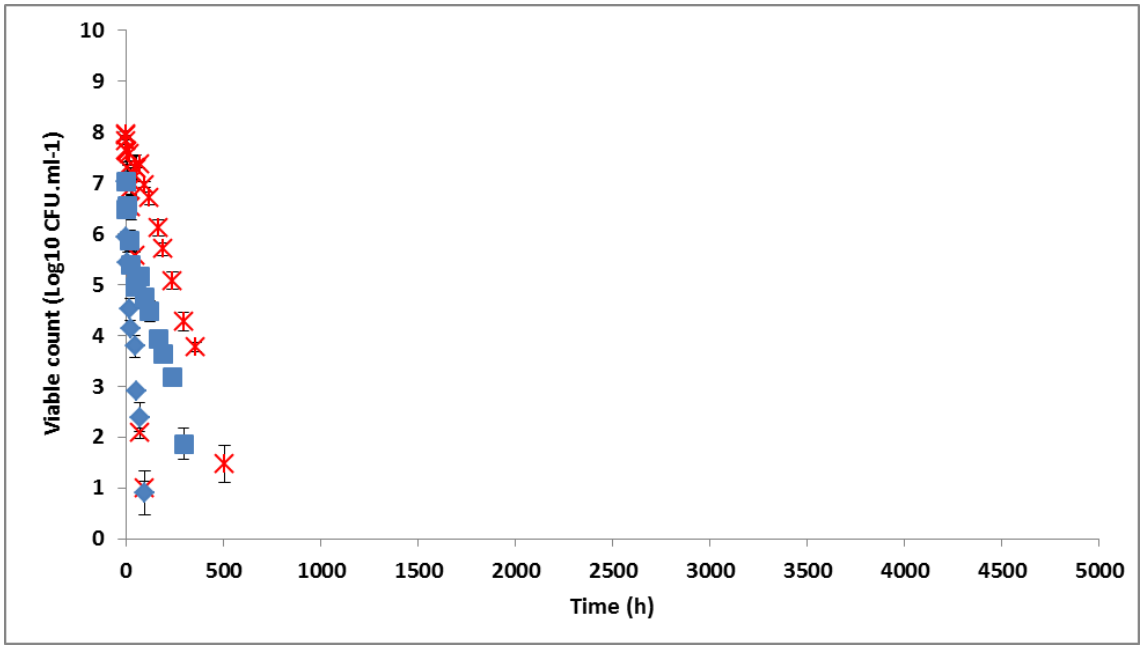

Figure 6.2d Inactivation data for *E. coli* in milk-based broth at a_w 0.945, pH 4.14, undissociated lactic acid 96.75 mM. *E. coli* R31 incubated at 15°C (✳) and 25°C (×); and *E. coli* M23 at 15°C (■) and 25°C (◆).

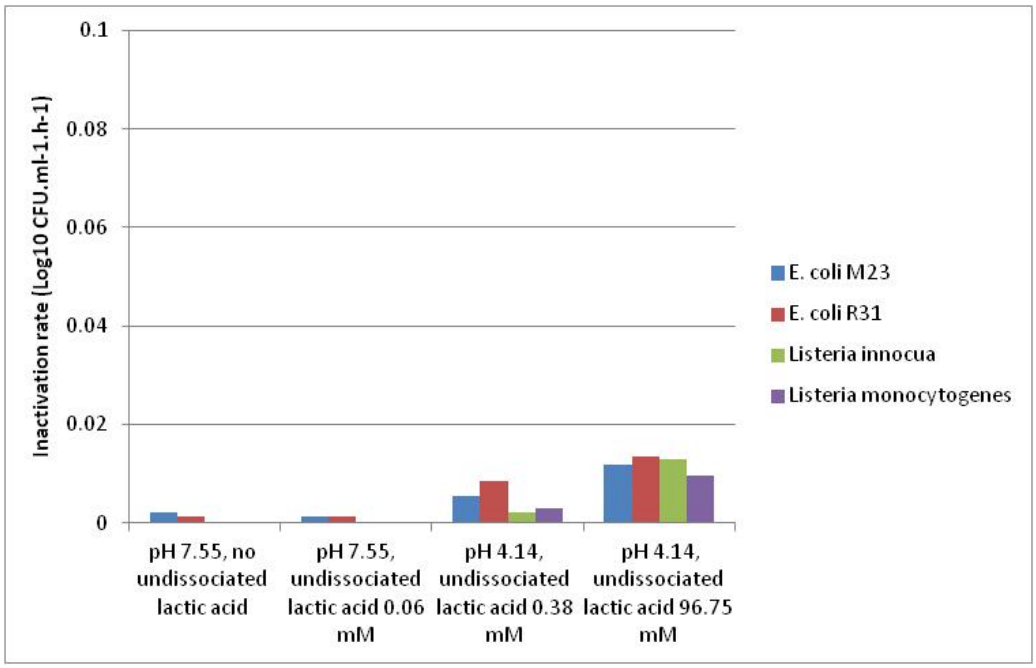

Figure 6.3a. Inactivation rates for both *Listeria* spp. and *E. coli* incubated in milk-based broth with different levels of pH and undissociated lactic acid at 15°C.

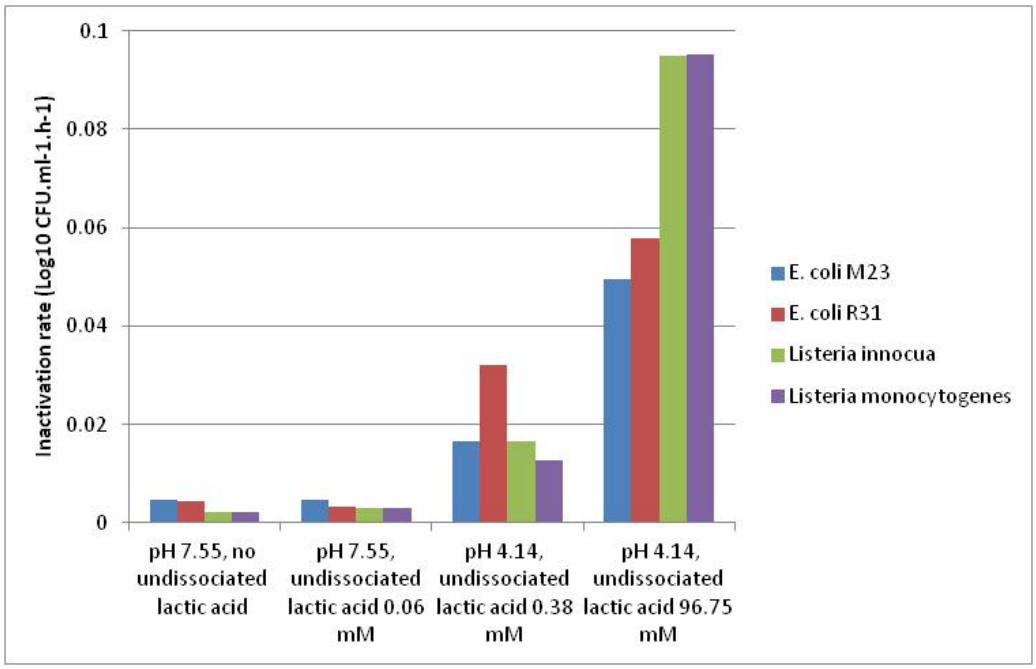

Figure 6.3b. Inactivation rates for both *Listeria* spp. and *E. coli* incubated in milk-based broth with different levels of pH and undissociated lactic acid at 25°C.

of undissociated lactic acid seems to have caused greater inactivation than in the complete absence of undissociated lactic acid. The effects of pH and undissociated lactic acid on bacterial inactivation observed indicate that rates of inactivation of both *Listeria* spp. and *E. coli* were affected by both pH and undissociated lactic acid concentration.

Figures 6.4a, b show that the absolute rates of inactivation of both *Listeria* spp. and *E. coli* in milk-based broths with different levels of pH and undissociated lactic acid were temperature-dependent. The apparent differences in inactivation pattern of *Listeria* spp. and *E. coli* are inconsistent with earlier trial to determine inactivation kinetics of the same challenge organisms in simulated cheese broths (*see* Section 2), but agree well with inactivation data from studies in Gouda cheese and Gouda cheese-like broth (Section 3). The temperature dependence of inactivation of *E. coli* observed in these experiments was consistent with results from earlier studies in cheeses and cheese-like broths (Sections 2 and 3)

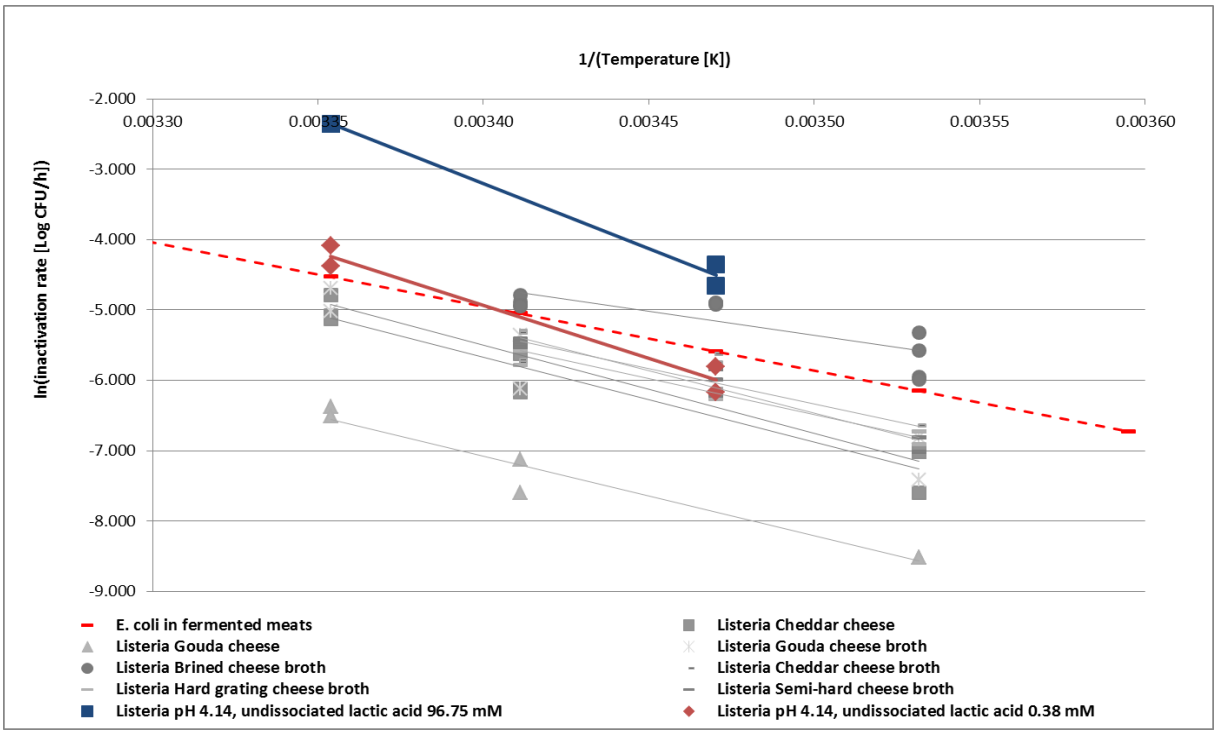

Figure 6.4a. Arrhenius plot of inactivation rates for *Listeria* spp. incubated in milk-based broths with different levels of pH and undissociated lactic acid compared to inactivation rates for *Listeria* spp. in cheeses and simulated cheese broths and those for *E. coli* in fermented meats. The red-dashed line is the model of McQuestin *et al.* (2009) for the inactivation rate of *E. coli* in fermented meats. The pale grey data is the models generated in the present study for *Listeria* inactivation in cheeses and cheese-like broths. Superimposed on these data are the inactivation data from the current work.

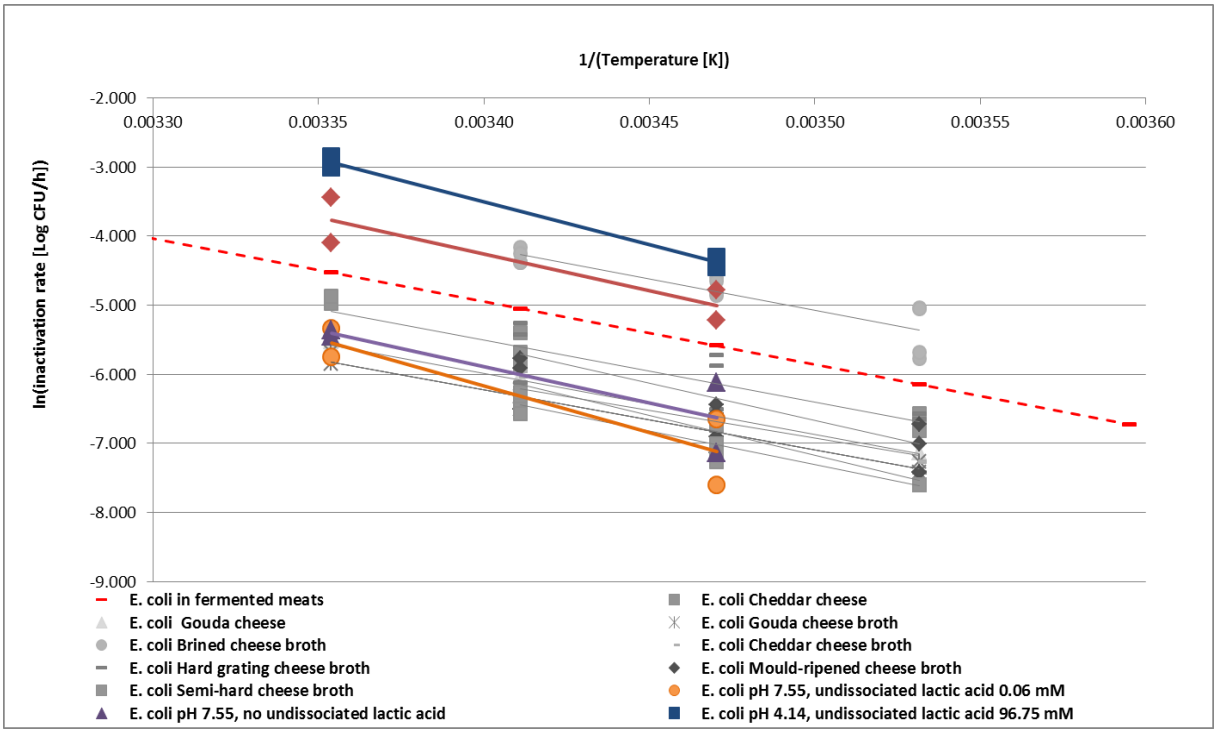

Figure 6.4b. Arrhenius plot of inactivation rates for *E. coli* incubated in milk-based broths with different levels of pH and undissociated lactic acid compared to inactivation rates for *E. coli* in cheeses and simulated cheese broths and those for *E. coli* in fermented meats. The red-dashed line is the model of McQuestin *et al.* (2009) for the inactivation rate of *E. coli* in fermented meats. The pale grey data is the models generated in the present study for *E. coli* inactivation in cheeses and cheese-like broths. Superimposed on these data are the inactivation data from the current work.

and those of McQuestin *et al.* (2009) in fermented meats, confirming that the effect of temperature on the inactivation of bacteria was not dependent upon the types of foods and/or media in which they were inoculated. Furthermore, the data reveal that the rates of inactivation of both *Listeria* spp. and *E. coli* in milk-based broths at high pH (e.g., > 5.0; regardless of undissociated lactic acid concentrations) were slower than average inactivation rates in fermented meats, whereas their inactivation rates were faster in milk-based broths at low pH (e.g., <5.0; regardless of undissociated lactic acid concentrations) than in fermented meats and other cheese-like broths. This is consistent with earlier observation in which the absolute rates of bacterial inactivation were faster in brined-cheese-like broths than in other types of foods and media. Brined cheeses typically have much lower pH and much higher undissociated lactic acid concentrations than the other cheese styles (Table 2.1).

These results suggest that while moderate levels of pH and/or undissociated lactic acid levels observed in most cheeses do not, of themselves, greatly accelerate inactivation rates of *E. coli* and *Listeria* spp. under inimical conditions, more extreme levels (*e.g.*, pH< 5, undissociated lactic acid >20 mM) may do so. Such effects were not detected in a literature review of inactivation rates

of *E. coli* in fermented meats based on scores of independent studies (McQuestin *et al*., 2009) but in that study very few data sets included pH <5 (lactic acid data were generally not available). However, FSANZ (2014) drew attention to the differences in the rates of inactivation reported for *E. coli* and *L. monocytogenes* at pH 3.5 and aw 0.900 by Ross *et al.* (2008) compared to inactivation rates observed in cheeses at various temperatures. The Ross *et al*. (2008) model predicts rates 5 – 10 times faster than observed. It is notable, however that the Ross *et al.* (2008) model predictions are much closer to the observed rates of inactivation in Feta-like broths, supporting the hypothesis that at very low pH levels, pH is an increasingly important additional predictor of pathogen inactivation rate.

Further studies to differentiate these effects were attempted but failed to produce results, predominantly because it was not possible to achieve high levels of undissociated lactic acid at near neutral pH, e.g., even at pH 5 (a level at which pH alone would be expected to cause ~10% growth rate inhibition [Ross *et al*., 2003; Mejlholm and Dalgaard, 2007]), to achieve 100mM undissociated lactic acid would require a total lactic acid concentration of ~4.5M (~400 $g.l^{-1}$).

7. Relationship of Results to the Current Scientific Literature

As part of the assessment of the reliability of the data generated from this study, and the interpretations based on them, this section presents a brief overview of those results in comparison to the existing scientific literature. Aspects considered are the physico-chemical characteristics of the cheeses, increases during acidification/curd formation, and inactivation and rates of inactivation during ripening.

FSANZ (2014) presented a comprehensive analysis of the relevant literature related to pathogen ecology in cheeses, and which directly addressed many of the topics indicated above. That document will be used as a baseline, but with additional comparison to selected, more recent, studies that are also pertinent.

7.1 Physico-chemical characteristics of cheeses.

As has been alluded to and demonstrated in previous Sections, there is a vast diversity of cheese styles and a workable (consistent) scheme of categorising cheeses has proven difficult to establish. Categorisations are often based on texture, production method, etc. but none is universally accepted. FSANZ (2014) adopted a categorisation based on production style including:

- Fresh (e.g., cottage cheese, quarg, queso fresco)
- Internally bacterially ripened cheese (e.g. Cheddar, Gouda, Wensleydale)
- Internal mould-ripened
- Surface mould-ripened
- Surface ripened (smear cheeses)

Those categories do not completely align with the categories used in this study, (i.e., which are: Brined, Cheddar ('hard'), Hard-grating, Internal-mould-ripened, Semi-hard, Soft, surface-ripened). Nonetheless, come categories are similar. FSANZ (2014) provided summary information on physico-chemical properties, including:

- Moisture content
- pH
- salt-in-moisture
- water activity

for each of these categories. Accordingly, a comparison of the results presented in Section 1 of this study, with the results of FSANZ' survey of reports in the published literature, is possible.

Table 7.1 summarises the data presented in FSANZ (2014; Figure 3) that corresponds to characteristics measured in this study (*see* Table 1.1). The data presented by FSANZ (2014) are summarised from five, relatively old, and potentially not very disparate, sources namely: Ruegg and Blanc (1997); Marcos *et al.* (1981); Marcos and Esteban, (1982); Marcos *et al.* (1990); Fernandez-Salguero *et al.* (1986). It should be noted that four of these five reports arise from the same group, based in Cordoba, Spain.

Table 7.1 Summary characteristics (median, with 25 to 75%-iles shown) of physico-chemical characteristics of selected cheese categories (derived from FSANZ, 2014).

Cheese category	% moisture average (range)	pH average (range)	water activity average (range)
internal bacterial ripened	38 35 to 41	5.5 5.4 to 5.7	0.96 0.95 to 0.97
internal mould ripened	43 40 to 45	6.0 5.7 to 6.2	0.92 0.91 to 0.94
surface mould ripened	51 50 to 53	6.7 7.3 to 6.3	0.97 0.965 to 0.98

By comparison, the data in Table 1.1 (obtained in this study) indicate that for cheddar style cheeses the average pH is 5.3 with an approximate 30 to 70%-ile range (approx. one standard deviation) from 5.1 to 5.5, water activity 0.94 with range 0.92 to 0.96 and moisture content from ~32 to 39%.

For surface mould-ripened cheeses, the average pH is 6.5 with an approximate 30 to 70%-ile range from 5.9 to 7.0, water activity 0.98 with range 0.97 to 0.99 and moisture content from ~44 to 53%. For internal mould-ripened cheeses, the average pH is 6.2 with an approximate 30 to 70% range from 5.3 to 7.1, water activity 0.93 with range 0.91 to 0.95 and moisture content average of 45%, with range from ~42 to 48%. Noting that the summary statistics used are not directly comparable (i.e. median *vs* mean, percentiles based on numbers of observations *vs.* percentiles from the modelled normal distribution) the data from the two sources seem to be essentially congruent, particularly given the geographic and temporal differences of the two studies.

Figure 4 from FSANZ (2014), which shows the correlation between pH and water activity of various cheeses, including variability between categories, is directly comparable to Figure 1.6 of this report. Both figures show a remarkably similar spread of pH and a_w values across all cheeses, although the spread for individual categories is not as similar for internal bacterial ripened. If however, the categories of Cheddar cheese and Semi-hard cheeses used in this report are considered equivalent to 'internal bacterially ripened' as used by FSANZ (2014) the results of the two surveys are closer.

There is a paucity of published data on important physico-chemical characteristics of cheeses that are expected to influence the potential for growth, as well as the growth rate, under conditions where growth is possible. FSANZ (2014) noted that data on water activity and organic acid concentrations, in particular, were lacking. In this regard, the results presented in Section 1 have considerably expanded the relevant body of knowledge by providing measurements of water activity and lactic and acetic acid for nearly 100 different commercial cheeses.

7.2 Effect of Starter Cultures and Rates of Acidification on Pathogen Growth

Studies by Park and Marth (1972), involving co-culture in skim milk of *Salmonella* Typhimurium with a variety of lactic acid bacteria starter cultures, showed that growth potential of Salmonella was strongly dependent on the starter culture used. The range of growth was from 1 to 5 $\log_{10}$ CFU increase. When the data were reanalysed by FSANZ (2014) as the observed maximum rate of acidification due to each starter culture, there was a strong negative correlation between maximum rate of acidification, and extent of increase in Salmonella before growth was completely inhibited. The analysis used the Torrestiani *et al.* (1994) equation to estimate the maximum rate of acidification. The Torrestiani *et al.* (1994) equation also estimates the initial and final pH of the co-culture, but those data were not presented in FSANZ (2014) so it was not possible to seek other correlations between change in pH and pathogen outgrowth. Conversely, the Torrestiani *et al.* (1994) equation was not applied to the data developed in the *current* study. Such an analysis, however, would provide additional insights about factors most highly correlated with minimising the growth of pathogens during milk tempering/acidification during cheese-making, and which could be exploited in the selection of starter cultures that would be most appropriate for use with raw milk cheese. In the study reported here, acidification rate was crudely estimated from the change in pH (initial – final) and the time taken for maximum acidification. In comparison to the Park and Marth (1972) study, however, the data in the current study are confounded by multiple strains of organism, each with their own growth rate/growth potential, so that the results of such an analysis are likely to be less clear than the Park and Marth (1972) study revealed. Nonetheless, even using the crude description of rate of acidification, rather than maximum rate of acidification, correlations between rate of acidification and total growth were revealed for most of the four challenge organisms and in both raw and pasteurised milk cheeses (*see* Figure 5.7b). The exceptions were *L. monocytogenes* in a pasteurised milk cheese, and *L. innocua* in raw milk cheese. These conclusions are based on small amounts of data (four points per organism and raw/pasteurised milks). Equally, however, correlations between growth amount and total pH *change* were also revealed in the analyses done in this project (*see* Figure 5.7a) and also should be considered based on fitting the Torrestiani *et al.* (1994) equation.

7.3 Cheese Challenge Studies

For the sake of comparison, it is assumed that the Feta cheese results from this study can be compared to the internal bacterial ripened cheese results (Papageorgiou and Marth, 1986), and that the double-cream Brie results can be compared to the surface-mould ripened cheese data (Pasteur Institute of Lille, 2001).

7.3.1 Internal bacterial ripened cheese

The data of Papageorgiou and Marth (1989) relate to Feta-style cheese, but here we make comparison with both the Feta and, for interest, Gouda results of this study. During fermentation the pH in Papageorgiou and Marth (1989) study fell to 4.7 over approximately 3 days, but took >12 hours to fall to pH 5. In our study, the pH of Feta cheese fell to 5.1 within ~8 - 10 hours (*see* Table

5.3a), and remained at that level. For the Gouda cheese, the pH fell to 5.4 within ~12 hours and remained at that level. The data in Table 1.1, and also Table 7.1 suggest that the Papageorgiou and Marth (1989) cheese achieved a pH characteristic of Feta, while our Feta did not. In Papageorgiou and Marth (1989) *Listeria* increased by ~2 $\log_{10}$CFU over 24 hours, of which 1 $\log_{10}$CFU was attributed to concentration of the cells into the curd. In our studies, the average increase of *Listeria* spp. in either raw or pasteurised milk Feta cheeses was 1.9 $\log_{10}$CFU (0.05 s.d.) and an average 1.53 $\log_{10}$CFU in Gouda cheeses (and up to 1.8 $\log_{10}$CFU). Thus, despite the lower final acidity, the time to reach a pH low enough to inhibit *Listeria* growth seems to have been similar in all cheeses, and with a similar total increase observed, though a large amount of variation in total growth achieved is evident.

7.3.2 Surface-mould ripened cheese

During fermentation the pH in surface-mould ripened cheese described in Pasteur Institute of Lille (2001) study fell to 4.6 in less than 12 h and to a lowest pH of 4.4 within ~1 day. In our study, the pH of double-cream Brie cheese fell to ~5.1 within ~24 hours (*see* Table 5.1a). The actual decrease may have been more rapid but could not be resolved from the times of observation/measurement, but was estimated by extrapolation of the available pH data to have occurred within ~12 h. The pH began to rise again after 10 – 15 days. The time to increase is consistent with that reported in The Pasteur Institute of Lille (2001) study. In that study, the surface pH was seen to rise quickly (within 5 to 10 days) to pH7, while the interior required approximately 35 days. The differences between surface and core in our trials were not so profoundly different, but the cheeses we made were relatively small, so that the amount of proteolytic enzymes penetrating the interior from the surface mould may have been greater. In the Pasteur Institute of Lille (2001) study, the increase in *L. monocytogenes* was from ~5 to ~5.9 $\log_{10}$CFU and considered by FSANZ (2014) to be due only to concentration, not growth. In our study, the average increase in *L. monocytogenes* was 1.25 (± 0.34 s.d.) $\log_{10}$CFU which is, again, relatively consistent with the observations of Pasteur Institute of Lille (2001).

7.3.3 Time to 'no net increase'

FSANZ (2014) reported the population dynamics of two strains of *Listeria monocytogenes* in a Feta style (high salt, internal bacterially ripened) raw milk cheese. The cheese was matured at 4°C in a 6% brine. The data presented, derived from Papageorgiou and Marth (1989) reveal significant strain differences in the kinetics with strain California showing only a slight lag of approximately 10 – 15 days before commencement of inactivation. The time before the population level of the California strain returns to the original inoculum level in the milk (i.e., no net change) is approximately 70 days. For strain Scott A, the lag time before inactivation commences was approximately 40 days, and time for the inoculated population to achieve 'no net change' approximately 120 days. The responses of *Listeria* spp. in raw-milk Feta cheese from the current study were shown in Figure 4.4b and reveal faster inactivation may occur. Both *L. monocytogenes* Scott A and *L. innocua* had lag times of approximately 10 days. The time taken to achieve 'no net increase' during maturation at 5°C is in

the range 60 to 100 days. These results are consistent with the behaviour of *L. monocytogenes* California, but faster than the inactivation kinetics of strain Scott A reported by Papageorgiou and Marth (1989).

Curiously, however, the results of Papageorgiou and Marth (1989) are very similar to our results for *Listeria* survival in Cheddar cheese (*see* Figure 4.3a). In our Cheddar-style cheese both *L. monocytogenes* Scott A and *L innocua* had lag times of approximately 45 days. The time taken to achieve 'no net increase' during maturation at 10°C, but without brine, is approximately 120 days. These results are consistent with the behaviour of *L. monocytogenes* Scott A in feta reported by Papageorgiou and Marth (1989). In our challenge trial involving *L. monocytogenes* Scott A in *pasteurised* milk Cheddar, however, the time for 'no net change' was 75 days, similar to the time observed by Papageorgiou and Marth (1989) for *L. monocytogenes* California.

FSANZ (2013) highlighted the strain differences observed in the Papageorgiou and Marth (1989) but our Cheddar data suggests that unexplainable differences, of the same magnitude, can occur as the strain differences observed by Papageorgiou and Marth (1989). Our Feta data produced faster inactivation rates of both *L. innocua* and *L. monocytogenes* than observed by Papageorgiou and Marth (1989) for *L. monocytogenes Scott* A, but results more similar to the results of Papageorgiou and Marth (1989) with *L. monocytogenes* Scott A. FSANZ (2013) also noted that the large strain differences observed including the possible presences of 'shoulders' (*i.e.*, lags before inactivation commences) makes estimating a time to no net change difficult. The comparison of results presented here suggests that the results of our experiments may be 'fail safe', and represent upper limits to survival times.

7.4 More Recent Studies

Various studies (*e.g*., Schvartzman *et al*., 2011) have commented on the relative paucity of comprehensive data on the fate of pathogens during cheese making.

A literature search was undertaken to identify other recent relevant challenge studies, not reviewed in FSANZ (2014), and that included close monitoring of changes in pathogen loads and physico-chemical characteristics throughout the entire cheese-making process for raw milk cheeses. Numerous challenge studies relevant to cheese were identified, but few satisfied the above criteria. The two most detailed and comprehensive studies found are Miszczycha *et al.* (2013) which included five styles of cheese (but two with lethal cooking steps) and four strains of shiga-toxin producing *E. coli* and D'Amico *et al.* (2010) which involved study of the population kinetics of three strains of *E. coli* O157:H7 in Gouda and stirred Cheddar.

The D'Amico *et al*. (2010) study, in response to criticisms of methodology for cheese challenge studies (see *e.g.*, Donnelly, 2001), used very low inoculum levels (~20 CFU.ml^{-1}). They reported an increase during tempering/acidification of ~10-fold, and that number then began to reduce during maturation. This increasing challenge organism concentration is less than in many other studies. Because of the low inoculum level, inactivation rates were difficult to calculate. Nonetheless, they reported that changes in pathogen levels observed throughout manufacture and aging did not significantly differ by cheese type. Moreover, D'Amico *et al.* (2010) found no strain differences: no

significant differences in mean counts between strains were found at each individual sampling point or during the 180-day aging period overall. Furthermore, probably because of the limited (quantitative) data, strong correlations between *E. coli* levels and physico-chemical characteristics of the cheese could not be established by D'Amico *et al.* (2010). Times for pathogen levels to achieve 'no net change' were 40 to 60 days, which is less than observed in most of the cheeses reported in the current study, but is most probably because the increase in pathogen levels during acidification/tempering was slight compared to observations in work reported here. The D'Amico *et al.* (2010) study included measurements of fat, pH, salt-in-moisture, and titratable acidity, but not organic acid concentrations.

Miszczycha *et al.* (2013) studied the population kinetics of four strains of STECs in five styles of raw milk cheeses. In cheeses that did not involve a cooking step, pathogens survived and the time to 'no net change' was in the range 80 -100 days in a 'Blue' style cheese, and much greater than 40 days in an uncooked, pressed curd, cheese made in 250 g portions. Strains differences were observed with both strains of *E. coli* O157:H7 growing less and surviving less well than other *E. coli* serotypes studied. Growth during initial stages of processing growth/concentration was in the range 2 to 3 $\log_{10}$CFU. During the challenge trials, pH, a_w and lactic acid concentrations were measured.

Maher *et al.* (2001) studied *E. coli* O157:H7 survival in raw milk smear cheeses. pH fell to 5.5 during acidification during which time *E. coli* grew by 1.9 $\log_{10}$CFU. Schvartzman *et al.* (2011) studied *L. monocytogenes* ecology during production of smear cheeses and reported 2 $\log_{10}$CFU increases during tempering/acidification. These levels of increase are consistent with those observed in our studies, but by comparison with other reports, also indicate the extent of variability in those in increases. Some of this variability may arise from the levels of background microbiota. Schvartzman *et al.* (2011) noted that in their studies higher background microbiota inhibited lactic acid bacteria which in turn slowed acid production and also resulted in higher final pH. In the studies conducted here, background microbiota levels in raw milks were in the range 2.5 to 4 $\log_{10}$CFU.ml^{-1}.

In conclusion, the results of the work undertaken in the study reported here do not seem inconsistent with trends reported by other workers and, as such, can be considered as equally reliable representations of the fate of *E. coli* and *Listeria* spp. in both raw and pasteurised milk cheeses, but subject to expressed caveats about strain to strain variation, reliability of measurements, and occasional unexplained differences in the dynamics of pathogen populations in apparently very similar cheeses. As such, the results in this report provide a valuable and substantial contribution to the body of knowledge on the topic, and which can assist in food safety risk management decisions about raw milk cheeses.

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APPENDIX 1. Methods of Physico-chemical Analyses of Cheese

Ninety-eight cheeses have been assessed in seven nominal categories, and from a wide range of manufacturers. All cheeses purchased were characterised in terms of:

- pH
- water activity
- water content
- lactic acid concentration
- acetic acid concentration
- using methods described below.

Additionally, the microscopic structure of some cheeses was visualised using scanning environmental electron microscopy (which is done under relatively low vacuum and involves minimal sample preparation) to better understand the physical structure of cheeses, particularly whether they are oil-in-water, or water-in-oil emulsions.

pH measurement

Samples (5 g) of each cheese were combined with 10 ml of distilled water. The mixture was stomached (Colworth Stomacher 400, A.J. Seward, UK) for 5 min. Thereafter, the pH of duplicates of each sample was measured using a calibrated digital pH meter and probe.

Water activity measurement

Cheese samples were chopped into small pieces to increase surface area and speed the rate of equilibration of humidity. Water activity was measured using an Aqualab CX2 dew-point instrument (Decagon Devices, Pullman, WA, USA). The reported water activity is derived from the average of triplicate measurements of duplicate samples.

Determination of total water content

Samples (10 g) of cheeses were placed in a drying oven set at 105°C. The sample was left to dry, and weighed daily until a constant weight was obtained, i.e. no further weight reduction. The water content of cheese was then estimated from the differences in weight before and after drying. The reported moisture content is based on the results of duplicate samples.

Determination of L-lactic acid and acetic acid content

Samples (2 g) of cheese were weighed out and transferred to a 50 ml falcon tube containing 30 ml of distilled water. This mixture was heated at 70°C with occasional shaking for 30 min and then mixed with approximately 20 ml of distilled water. After holding on ice for 30 min, the mixture was filtered. An appropriate volume of sample was assayed for L-lactic acid concentration using a commercial test kit, (K-LATE, Megazyme International Ireland Limited, Ireland), according to the manufacturer's

instructions. Similarly, acetic acid concentration was determined using a commercial test kit, (K-ACETRM, Megazyme International Ireland, Limited), according to the manufacturer's instructions. Reported organic acid concentrations are based on the results of analyses of duplicate samples.

Environmental scanning electron microscopy

The microstructure of various cheese samples/styles was examined by environmental scanning electron microscopy using an FEI Quanta MLA 600 environmental scanning electron microscope (ESEM). Cheese samples were cut into thin slices, sputter-coated, and pre-cooled. The ESEM chamber was then evacuated to 4 Torr. A voltage of 25.0 kV was used. Areas of interest were identified and electron micrographs taken to record the observed 'ultrastructure'.

APPENDIX 2. Detailed results of analyses of cheeses

Brand/Processor and additional information	Expiry date	Type	pH	Water activity	Water Content (% w/w)	Lactic acid (ppt)	Acetic acid (ppt)
Adelaide Hills, hand made by Woodside cheese wrights	NA	Brined cheese	4.63	0.971	50.30	0.35	0.05
Australian style, Coles	13.03.2013	"	4.17	0.963	51.35		
Danish style, Josephine's, Denmark	18.02.2013	"	4.49	0.966	58.80		
Dodoni	08.07.2012	"	4.30	0.966	54.40	0.69	0.04
Dodoni, Greek Feta. Made from pasteurized sheep's and goat's milk	20.07.2012	"	4.35	0.968	50.70		
Hill Street Grocer	Dated packed only 28.02.2012	"	4.53	0.955	50.40	0.50	0.10
Lemnos	16.12.2012	"	4.14	0.971	54.10	1.14	0.06
Lemnos	10.11.2012	"	4.49	0.965	48.30	0.31	0.03
Lemnos	04.11.2012	"	4.23	0.967	55.05	0.50	0.03
Made from pacteurised sheep's and goat's milk, MEVGAL	19.10.2012	"	4.43	0.971	55.40	0.61	0.16
Mayers Danish Fetta	17.4.2013	"	4.61	0.933	57.90	1.48	0.00
Ultimate Feta Tasmania	14.6.2013	"	4.61	0.979	59.60	2.53	0
Ashgrove TAS farm cheese aged for 12 months	22.06.2012	Cheddar cheese	5.27	0.949	35.80	0.54	0.05
Ashgrove TAS farm cheese aged for up to 15 months	19.07.2012	"	5.56	0.918	28.20	0.48	0.23
Bega Cheese Limited	03.01.2013	'	5.45	0.956	36.45	0.67	0.02
Beqa, TASTY	18.10.2012	"	5.14	0.958	36.85		
Coles, aged up to 14 months	13.10.2012	"	5.01	0.947	35.15		
Cracker Barrel, Extra sharp, aged up to 20 months	10.08.2012	"	5.25	0.936	36.35		
Cracker Barrel, Special reserve, aged up to 36 months	20.08.2012	"	5.42	0.916	34.45		
Cracker Barrel, Vintage light (25% less fat), aged up to 15 months	06.08.2012	"	5.36	0.956	40.25		
Hand price, Hill Street Grocer	Dated packed only 10.02.2012	"	5.44	0.903	29.60	0.55	0.19
Hill Street Grocer	Dated packed only 27.02.2012	"	5.06	0.948	35.20	1.27	0.05
Jindi	24.07.2012	"	5.02	0.955	35.45	0.84	0.06
Kraft, Natural cheese	28.08.2012	"	5.77	0.955	35.40	0.52	0.19
Mainland extra tasty cheddar 18 months	28.2.2013	"	5.33	0.939	37.95	1.76	0.12

Brand/Processor and additional information	Expiry date	Type	pH	Water activity	Water Content (% w/w)	Lactic acid (ppt)	Acetic acid (ppt)
Mainland Vintage cheddar 24 months	28.3.2013	Cheddar cheese	5.21	0.940	36.05	0.96	0.02
National Foods Limited	09.09.2012	"	5.29	0.956	41.05	0.72	0.02
South Cape	07.09.2012	"	5.23	0.951	35.20		
Hill Street Grocer, hard Italian cheese	Dated packed only 21.02.2012	Hard grating cheese	5.10	0.892	36.30	0.96	0.07
Zanetta	27.2.2013	"	5.47	0.912	35.65	1.11	0.14
Parmesan, Wattle valley	21.3.2013	"	5.29	0.885	32.95	1.18	0.15
Pecorino South Cape	NA	"	5.32	0.918	37.05	1.51	0
Perfect Italiano Fonterra	NA	"	5.39	0.910	38.65	0.96	0.07
Hill Street Grocer	Dated packed only 13.05.2012	"	5.71	0.888	36.65	0.39	0.04
Castello	5.09.2012	Mould-ripened cheese	5.15	0.910	45.30	0.55	0.10
Castello	13.08.2013	"	5.26	0.907		0.60	0.07
Castello		"	5.32	0.922		0.66	0.04
Hand Price, Hill Street Grocer	Dated packed only 04.05.2012	"	6.80	0.930	41.10	0.05	0.08
Hill Street Grocer	Dated packed only 11.05.2012	"	7.18	0.945	49.95	0.25	0.07
Hill Street Grocer, made from ewe's milk, France	Dated packed only 08.05.2012	'	6.20	0.924	46.90	0.25	0.12
Long Clawson Dairy Ltd., Product of United Kingdom	30.06.2012	"	6.07	0.955	42.65		
Mainland, blue cheese	26.10.2012	"	5.88	0.913	42.45	0.92	0.34
Mr Bennetts Blue Ashgrove	13.02.2013	"	6.98	0.945		0.19	0.05
Mr Bennetts Blue Ashgrove	14.10.2012	"	7.47	0.954	45.65	0.01	0.04
The Diary Viking Denmark	NA	"	5.11	0.923	48.25	1.70	0.05
unknown	NA	"	7.52	0.942	42.20		
Red leicester style	14.06.2012	'Others'	5.65	0.951	36.85	0.59	0.20
Lemnos	16.05.2012	"	5.13	0.990	63.45	0.34	0.05
Mainland, Mild flavour	28.08.2012	"	5.25	0.957	37.50		
Kraft, Natural cheese	11.10.2012	"	5.38	0.966	37.50		
Woolworths, New Zealand cheese	28.08.2012	"	5.40	0.957	37.05		
Emborg	14.2.2012	"	5.68	0.977	38.35	0.24	0.32

Brand/Processor and additional information	Expiry date	Type	pH	Water activity	Water Content (% w/w)	Lactic acid (ppt)	Acetic acid (ppt)
Ashgrove Claus' Havarti	NA	Semi-hard cheese	5.47	0.943	46.35	0.83	0
Frico Mild Dutch Edam	24.5.2013	"	5.56	0.952	45.45	1.18	0
Hand Price, Hill Street Grocer	Dated packed only 17.12.2012	"	5.44	0.952	38.20	0.29	0.05
Hill Street Grocer	Dated packed only 31.12.2011	"	5.25	0.976	38.50		
Mainland	NA	"	5.29	0.963	41.35	1.24	0.04
Mainland, straight from the block	12.11.2012	"	5.38	0.956	41.05	1.44	0.03
Mayers	8.5.2013	"	5.83	0.964	43.20	0.00	0.43
Pantalico	14.2.2013	"	5.62	0.972	46.10	0.94	0.08
Perfect Italiano Fonterra	?.?.2013	"	5.64	0.973	46.90	0.64	0.04
Product of Australia, South Cape	11.12.2012	"	5.74	0.965	42.85		
Product of Australia, South Cape	04.12.2012	"	5.71	0.974	40.70		
Product of Australia, South Cape	03.09.2012	"	5.31	0.951	42.55		
Product of Australia, South Cape	11.12.2012	"	5.99	0.959	39.15	0.27	0.04
Product of Australia, South Cape	28.02.2013	"	5.86	0.970	37.90	0.40	0.05
Product of England, Singletans	03.11.2012	"	5.17	0.961	36.25		
South cape	3.04.2012	"	5.29	0.961	37.65	0.84	0.04
South Cape	23.05.2012	"	5.71	0.964	39.80	0.71	0.04
Tasmanian Heritage	2.1.2013	"	5.85	0.964	38.45	0.62	0.23
type of swiss cheese, Hill Street Grocer	Dated packed only 11.05.2012	"	5.68	0.961	41.00	0.64	0.08
Woolworths	04.09.2012	"	5.52	0.962	46.70	2.19	0.05
Woolworths	02.10.2012	"	5.40	0.962	44.65	1.53	0.06
Woolworths select Gouda slices	7.5.13	"	5.64	0.955			0.11
Australian Gold	07.12.2012	Soft, surface-ripened cheese	6.14	0.989	57.05	0.10	0.04
Australian Gold	12.01.2013	"	5.94	0.985	52.80	0.24	0
Australian Gold	10.04.2013	"	6.04	0.987	52.60	0.10	0
Australian Gold	09.03.2013	"	6.31	0.986	53.60	0.09	0.00
Australian Gold	18.01.2013	"	5.74	0.985	52.90	0.15	0.02
Fresh Australian, Unicorn cheese	22.05.2012	"	7.44	0.974	48.25	0.01	0.11

Brand/Processor and additional information	Expiry date	Type	pH	Water activity	Water Content (% w/w)	Lactic acid (ppt)	Acetic acid (ppt)
Hill Street Grocer	Dated packed only 11.05.2012	Soft, surface-ripened cheese	6.22	0.982	47.90	0.03	0.02
Friendship French Brie	30.9.2012	"	6.84	0.976	46.40	0.25	0
Hand Price, Hill Street Grocer	Dated packed only 14.05.2012	"	5.94	0.979	44.55	0.04	0.02
Jindi	29.9.2012	"	7.35	0.974	48.70	0.02	0.04
King Island Dairy Capewickham Double Brie	31.10.2012	"	5.91	0.974	47.35	0.14	0.02
King Island Dairy Capewickham Double Brie	22.01.2013	"	6.35	0.977		0.04	0.04
King Island Dairy Capewickham Double Brie	18.01.2013	"	6.63	0.964		0.01	0.04
King Island Dairy Lighthouse Blue Brie	15.10.2012	"	6.66	0.959	42.60	0.04	0.02
Mainland	28.08.2012	"	5.76	0.990	55.20	0.20	0.04
Mainland	28.08.2012	"	6.00	0.987	54.15	0.15	0.03
Red Square washed rind Tasmanian Heritage	18.10.2013	"	6.14	0.973	45.20	0.01	0.03
Red Square washed rind Tasmanian Heritage	11.10.2013	"	6.38	0.975	40.35	0.02	0.04
Southcape Brie	1.10.2012	"	7.55	0.971	47.70	0.03	0.04
Southcape Brie	03.02.2013	"	6.26	0.970		0.03	0.04
Southcape Brie	26.01.2013	"	6.50	0.962		0.02	0.04
Tasmanian Heritage	30.05.2012	"	6.97	0.977	41.70	0.01	0.07
Tasmanian Heritage	19.01.2013	"	7.32	0.973		0.00	0.05
Tasmanian Heritage	04.02.2013	"	6.63	0.974		0.03	0.04

APPENDIX 3. Inactivation rates of bacteria in broths intended to emulate cheese.

Inactivation rates of *Listeria* spp. in simulated cheese broths at three different temperatures.

Bacterial strains	Inactivation rates (Log_{10} CFU/ h)		
	10°C	15°C	20°C
Brined cheese-like broth			
L. innocua	0.0026	0.0074	0.0080
L. monocytogenes Scott A	0.0044	0.0075	0.0072
Cheddar cheese-like broth			
L. innocua	0.0012	0.0034	0.0050
L. monocytogenes Scott A	0.0012	0.0022	0.0033
Hard grating cheese-like broth			
L. innocua	0.0012	0.0020	0.0035
L. monocytogenes Scott A	0.0011	0.0021	0.0044
Mould-ripened cheese-like broth			
L. innocua	NI[a]	0.0010	0.0029
L. monocytogenes Scott A	NI[a]	0.0023	0.0031
Semi-hard cheese-like broth			
L. innocua	0.0009	0.0023	0.0041
L. monocytogenes Scott A	0.0011	0.0029	0.0042

a. NI = no inactivation is observed within 435 days.

Inactivation rates of *E. coli*. in simulated cheese broths at three different temperatures.

Bacterial strains	Inactivation rates (Log_{10} CFU/ h)		
	10°C	15°C	20°C
Brined cheese-like broth			
E. coli M23	0.0033	0.0079	0.0125
E. coli R31	0.0065	0.0098	0.0149
Cheddar cheese-like broth			
E. coli M23	0.0007	0.0011	0.0025
E. coli R31	0.0005	0.0008	0.0022
Hard grating cheese-like broth			
E. coli M23	0.0014	0.0031	0.0048
E. coli R31	0.0006	0.0013	0.0021
Mould-ripened cheese-like broth			

E. coli M23	0.0011	0.0015	0.0029
E. coli R31	0.0006	0.0009	0.0015
Semi-hard cheese-like broth			
E. coli M23	0.0005	0.0007	0.0018
E. coli R31	0.0005	0.0009	0.0015

APPENDIX 4. Inactivation rates of bacteria in cheese products and simulated cheese broths.

Inactivation rates of *Listeria* spp. and *E. coli* in Gouda cheese and Gouda cheese-like broth at three different temperatures.

Bacterial strains	Inactivation rates (Log_{10} CFU/h)		
	10°C	15°C	20°C
L. innocua			
Gouda cheese	0.0002	0.0005	0.0017
Gouda cheese-like broth	0.0011	0.0047	0.0092
***L. monocytogenes* Scott A**			
Gouda cheese	0.0002	0.0008	0.0015
Gouda cheese-like broth	0.0006	0.0022	0.0066
***E. coli* M23**			
Gouda cheese	0.0008	0.0024	0.0040
Gouda cheese-like broth	0.0007	0.0018	0.0029
***E. coli* R31**			
Gouda cheese	0.0008	0.0020	0.0038
Gouda cheese-like broth	0.0006	0.0015	0.0034

APPENDIX 5. Inactivation rates of bacteria as a function of pH and lactic acid concentration.

Inactivation rates of *Listeria* spp. in milk-based broths at two different temperatures.

Bacterial strains	Inactivation rates (Log_{10} CFU/ml/h)	
	15°C	**25°C**
pH 7.55, no undissociated lactic acid		
L. innocua	NA[a]	0.0022
L. monocytogenes Scott A	NA	0.0021
pH 7.55, 0.06 mM undissociated lactic acid		
L. innocua	NA	0.0029
L. monocytogenes Scott A	NA	0.0030
pH 4.14, 0.38 mM undissociated lactic acid		
L. innocua	0.0021	0.0167
L. monocytogenes Scott A	0.0030	0.0126
pH 4.14, 96.75 mM undissociated lactic acid		
L. innocua	0.0128	0.0950
L. monocytogenes Scott A	0.0095	0.0951

[a.] NA = not available because rates were not able to be determined during the time course of the experiment.

APPENDIX 5 (cont.). Inactivation rates of bacteria as a function of pH and lactic acid concentration.

Inactivation rates of *E. coli* in milk-based broths at two different temperatures.

Bacterial strains	Inactivation rates (Log_{10} CFU/ml/h)	
	15°C	25°C
pH 7.55, no undissociated lactic acid		
E. coli M23	0.0022	0.0047
E. coli R31	0.0008	0.0043
pH 7.55, 0.06 mM undissociated lactic acid		
E. coli M23	0.0013	0.0048
E. coli R31	0.0005	0.0032
pH 4.14, 0.38 mM undissociated lactic acid		
E. coli M23	0.0054	0.0166
E. coli R31	0.0084	0.0320
pH 4.14, 96.75 mM undissociated lactic acid		
E. coli M23	0.0118	0.0494
E. coli R31	0.0135	0.0578

APPENDIX 6. Physico-chemical properties of cheeses during production and maturation.

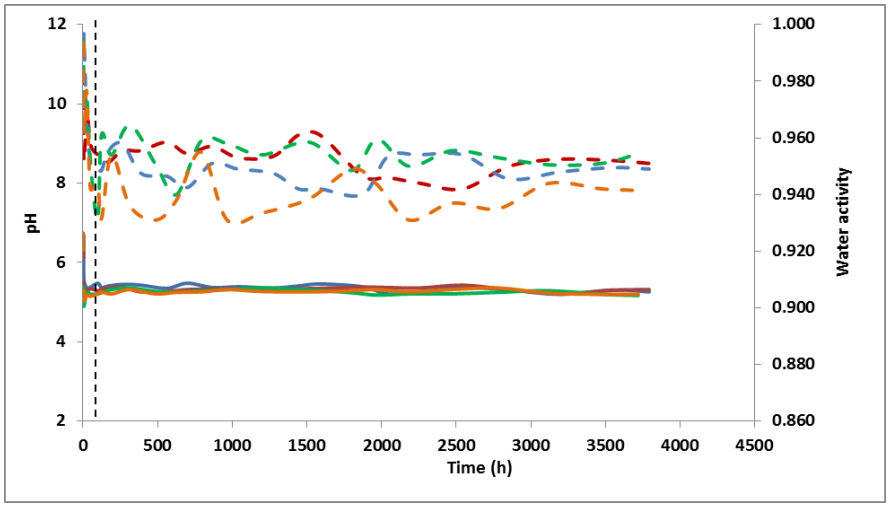

Fig. A6.1. Changes in pH (solid lines) and water activity (dashed lines) during the making of Cheddar-style cheeses made from raw milk (blue and green) and pasteurised (red and orange) milk. Data were collected from two different batches of cheeses. The dashed line separates the initial cheese formation (left side) from the maturation period (right side).

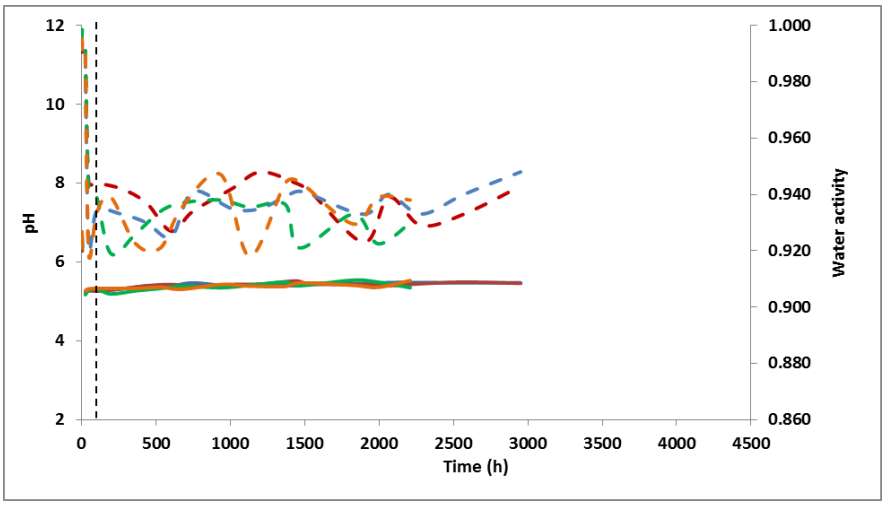

Fig. A6.2. Changes in pH (solid lines) and water activity (dashed lines) during the making of Gouda-style cheeses made from raw milk (blue and green) and pasteurised (red and orange) milk. Data were collected from two different batches of cheeses. The dashed line separates the initial cheese formation (left side) from the maturation period (right side).

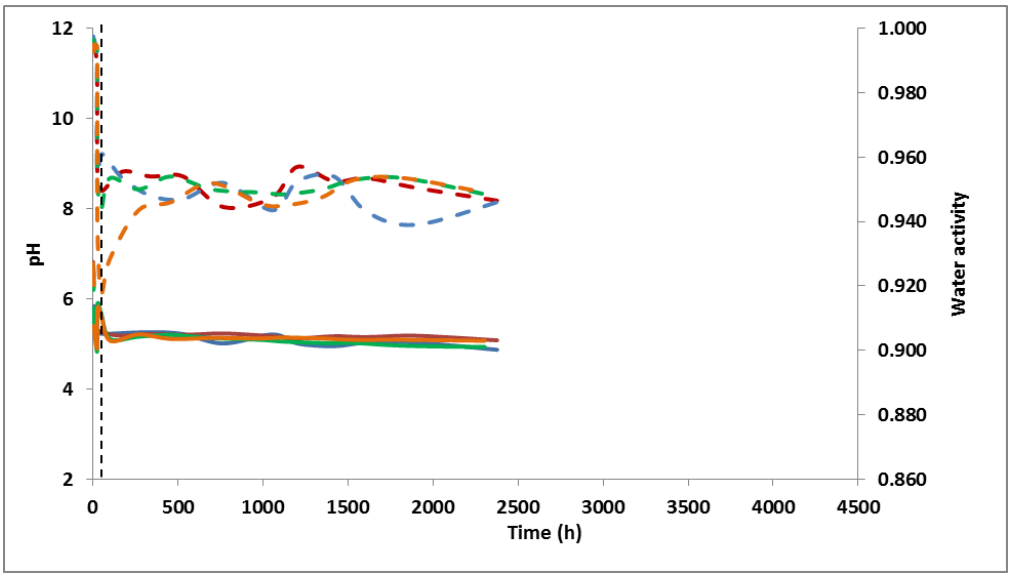

Fig. A6.3. Changes in pH (solid lines) and water activity (dashed lines) during the making of Feta-style cheeses made from raw milk (blue and green) and pasteurised (red and orange) milk. Data were collected from two different batches of cheeses that were kept at 15°C during maturation period. The dashed line separates the initial cheese formation (left side) from the maturation period (right side).

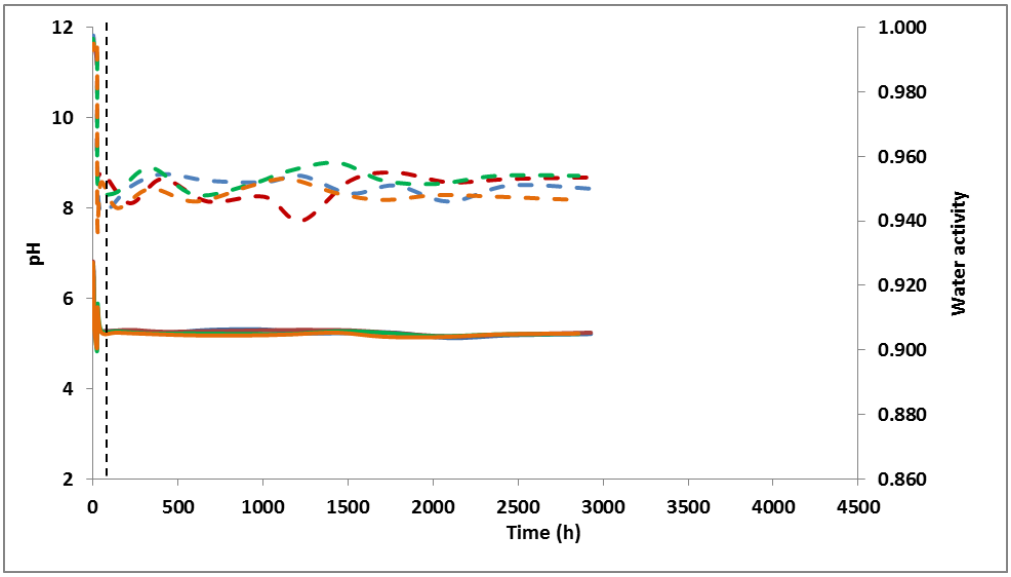

Fig. A6.4. Changes in pH (solid lines) and water activity (dashed lines) during the making of Feta-style cheeses made from raw milk (blue and green) and pasteurised (red and orange) milk. Data were collected from two different batches of cheeses that were kept at 5°C during maturation period. The dashed line separates the initial cheese formation (left side) from the maturation period (right side).

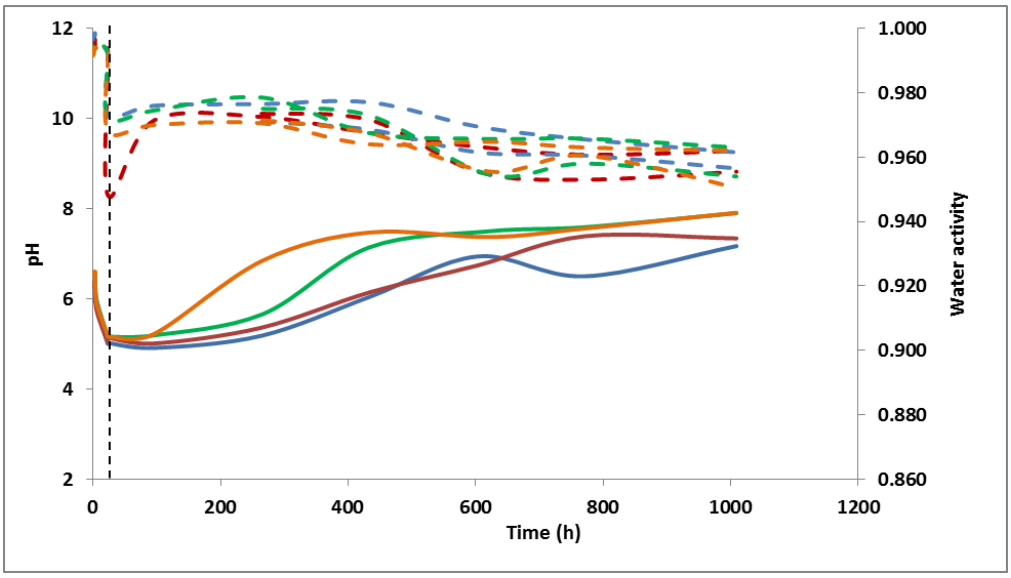

Fig. A6.5. Changes in pH (solid lines) and water activity (dashed lines) during the making of Double-cream Brie-style cheeses made from raw milk (blue and green) and pasteurised (red and orange) milk. Data were collected from two different batches of cheeses. The dashed line separates the initial cheese formation (left side) from the maturation period (right side).

APPENDIX 7. Inactivation rates of bacteria during maturation of cheeses.

Inactivation rates of *Listeria* spp. during maturation of different styles of cheeses.

Descriptions	*L. innocua*		*L. monocytogenes* ScottA	
	Raw milk	Pasteurised milk	Raw milk	Pasteurised milk
Wensleydale cheese, 15°C[a]	NA[b]	NA	NA	NA
Cheddar cheese, 10°C	0.0013	0.0015	NA	0.0022
Gouda cheese, 15°C	0.0016	0.0021	0.0028	0.0031
Feta cheese, 15°C	0.0035	0.0039	0.0043	0.0038
Feta cheese, 5°C	0.0014	0.0016	0.0010	0.0010

[a.] Temperature at which cheese was kept during maturation.

[b.] NA = not available because rates were not able to be determined during the time course of the experiments

Inactivation rates of *E. coli* during maturation of different styles of cheeses.

Descriptions	*E. coli* M23		*E. coli* R31	
	Raw milk	Pasteurised milk	Raw milk	Pasteurised milk
Wensleydale cheese, 15°C[a]	0.0009	0.0012	0.0008	0.0007
Cheddar cheese, 10°C	0.0012	0.0015	NA[d]	0.0020
Gouda cheese, 15°C	0.0034	0.0034	0.0031	0.0039
Feta cheese, 15°C	0.0039	0.0042	0.0040	0.0039
Feta cheese, 5°C	0.0021	0.0016	0.0010	0.0010

[c.] Temperature at which cheese was kept during maturation.

[d.] NA = not available because rates were not able to be determined during the time course of the experiments

APPENDIX 8. Cheese-making Protocols and Methods of Analysis

All cheeses were made at the laboratory of the Food Safety Centre, University of Tasmania (AUS) by an experienced home cheese maker. Specialised advice was also obtained from Mr. Ashley McCoy, owner and cheese-maker at Wicked Cheese, Cambridge, Tasmania and, on occasion, by independent colleagues whom he consulted. Mr. McCoy is a professional cheese maker and has received a number of awards for his cheeses. For further information see: http://www.wickedcheese.com.au/cheesemaker.php.

Bacterial Strains

Two strains of *E. coli* (M23 and R31) and two species of *Listeria* (*L. innocua* and *L. monocytogenes* Scott A) obtained from the culture collection of the Food Safety Centre, University of Tasmania (AUS) were used as a challenge organism in this study.

Commercially available stater cultures were obtained from CheeseLinks (http://cheeselinks.com.au/). Table A1 describes each type of stater cultures used in this study.

Table A8.1. Starter cultures blends used in this study.

Starter culture	Bacterial composition
Type A Farmhouse Starter	*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *Lactis biovar diacetylactis* and *Streptococcus themophilus*
Type B Starter	*Lactococcus lactis* subsp. *Lactis*, *Lactococcus lactis* subsp. *cremoris* and *Lactoccocus lactis* subsp. *Lactis biovar diacetylactis*
Type E Starter	*Streptococcus thermophilus*

Preparation of Challenge Organisms

Cultures previously maintained at -80°C were re-cultivated by streaking onto nutrient agar and incubating at 37°C for 24 h. To prepare inocula, stationary phase cells (i.e. approximately 10^{8-9} CFU/ml) were prepared by inoculating cells (2 single colonies for *E. coli* or 10 single colonies for *Listeria* spp.) into 50 ml of milk-based broth (1% full cream milk powder, 0.5% peptone and 0.3% yeast extract) and incubated at 30°C for 24 h. Inocula were prepared by centrifugation at 4,800rpm for 15 min, operating at room temperature. The pellet was re-suspended with 1 ml of 0.1% peptone water. This bacterial suspension was then used as an inoculum to experimentally contaminate milk before cheese manufacture.

Preparation of Milk

Each style of cheese, other than Wensleydale which was made with goat's milk, was made from whole cow's milk. Raw, fresh milk (approximately 10 L in total) was collected from a local farm on the day of cheese manufacture. The milk was thoroughly mixed prior to use to avoid any potential influences that variations in the composition of the milk from each source could have had on the experiment. After mixing, the milk was separated into two 10L lots and placed in sterile stainless steel pots. Milk was then divided into two lots of 5 L and transferred to sterile 10-liter vats. One of these lots was subjected to pasteurisation (68°C, 1 min). Both pasteurised (after pasteurisation) and raw milk were cooled or warmed to the temperature appropriate for making each type of cheeses (*see below*).

Temperature Control

Milk was held in large stainless steel saucepans contained within a larger saucepans containing water to buffer temperature changes. Temperatures were monitored with digital thermometers. Saucepans were placed on free-standing induction heating hot plates which enabled near to instantaneous input, or cessation of heating, and enabled very good temperature control.

Equipment Sterilisation and Cleaning

To minimise the additional contamination of the cheeses made all pots and utensils were initially scrubbed with a general purpose cleaning agent (domestic dishwashing liquid) then thoroughly rinsed with distilled water. Any pots expected to come into direct contact with the milk during the experiment were also exposed to a germicidal UV light (385 nm) for 15 minutes prior to use. The utensils were also subjected to a 16-24 hour soak in a domestic hypochlorite solution (White King, Regular Premium Bleach: Strong, at a ratio of 1:20) then rinsed with sterile distilled water before use.

After use, all pots were washed with a commercial hypochlorite solution (White King, Regular Premium Bleach: Strong, at a ratio of 1:20) and all utensils were placed to soak in a hypochlorite solution (1:20) for 16-24 hours. Any remnants of cheese were removed by scrubbing with a general purpose cleaning agent. After treatment all were rinsed with distilled water. Containers used to hold or transport the milk and/or whey were cleaned, both prior and after use with a hypochlorite solution (at a ratio of 1:20) and rinsed with distilled water.

The waste whey was disinfected by heating in a pot until boiling for 5 minutes. The disinfected whey solution was cooled and disposed of via the normal, municipal, sewage system.

Cheddar Cheese

Calcium chloride, ($CaCl_2$) was added to both pasteurised and raw milk at 32°C to achieve 100 mg/L, in addition to Type A stater (2 mg/L) and target bacteria (a mixture of one strain of *E. coli* and one species of *Listeria* in a ratio of 1:1; at approximately $10^{6\text{-}7}$ CFU/ml). The mixture was then held at

32°C for 1 h, and "vegetarian"[3] rennet (Chymax plus, 40 IMCU/L) then added to induce coagulation. After leaving for 35 min, the resulting coagulum was cut into small cube-shape particles and allowed to rest for 5 min. The curd-whey mixture was then stirred while its temperature was raised to 38°C over a period of 30 min. Stirring was continued for another 1 h. Thereafter, the whey was drained off, and the remaining curd stirred until it achieved pH 5.30 ± 0.05. The curd was milled and mixed with cooking salt (15 g in total). The salted curd was transferred to a plastic hoop lined with sterile disposable cheese cloth. The curd was then pressed with a 10 kg weight at 27°C for 20-22 h. Cheese was removed from the hoop and cut into 24 equal pieces of cheese (20-30 g) before they were vacuum sealed in polyethylene bags. All cheeses were stored at 10°C for maturation (at least six months).

Two batches of each raw and pasteurised milk cheddar were prepared, each containing a different combination of strain of *E. coli* or species of *Listeria*. Samples were taken for microbiological and chemical analyses, as described below, at various times during the cheese making and maturation process.

Gouda Cheese

Both pasteurised and raw milk at 32°C were combined with $CaCl_2$ (100 mg/L), Type B stater (2 mg/L) and challenge bacteria (a mixture of one strain of *E. coli* and one species of *Listeria* in a ratio of 1:1; approximately 10^{6-7} CFU/ml). After keeping for 1 h, vegetarian rennet (50 IMCU/L) was added and left for 40 min. The resulting coagulum was cut in cubes, allowed to rest for 5 min, and then stirred for 40 min. Thereafter, approximately 2.5 L of the whey was removed (i.e., half of the original milk level), and approximately 1 L of sterile tap water (pre-warmed at 60°C) was added. The temperature was then raised to 38°C, and stirring was continued for approximately 30 min. The whey was removed again to the point where it still just covered the surface of the curd. The curd was prepressed with light pressure (approximately 1.5 Kg weight) under the whey for 15 min. The whey was then drained, and the curd was placed in a plastic hoop and pressed with a 5 kg weight at 27°C for 20-22 h. Cheese was removed from the hoop and divided into 24 equal pieces of cheese (20-30 g). All cheeses were transferred to a 20% brine solution (1 L) for 15 min at room temperature, and allowed to dry in a 20°C incubator for 22 h, and then vacuum sealed. Cheeses were left to mature at 15°C for at least two months.

Two batches of each raw and pasteurised milk Gouda were prepared, each containing a different combination of strain of *E. coli* or species of *Listeria*. Samples were taken for microbiological and chemical analyses, as described below, at various times during the cheese making and maturation process.

Feta Cheese

Both pasteurised and raw milk at 34°C were combined with $CaCl_2$ (100 mg/L), Type A stater (2 mg/L), lipase (10 mg/L) and target bacteria (a mixture of one strain of *E. coli* and one species of *Listeria* in a

[3] "Vegetarian" rennet is produced from genetically modified bacteria containing the gene for chymosin B in fermentation cultures. The fermentation broth is processed to extract the chymosin, and excludes the microbial cells. Because it is not derived from animals, this product is marketed as 'vegetarian' rennet.

ratio of 1:1; approximately 10^{6-7} CFU/ml). The mixture was left for 1 h, and "vegetarian" rennet (40 IMCU/L) was added. After incubation for 1 h, the coagulum was cut into small cubic shaped pieces, and allowed to rest for 30 min. The curd-whey mixture was then stirred every half-hour over a period of 90 min before the whey was drained off. The remaining curd was transferred to a perforated hoop and turned after 10 min, 30 min, 1 h and 2 h. The curd was allowed to dry in a 20°C incubator for 20-22 h. Thereafter, cheese was removed from the hoop and cut into 24 equal pieces of cheese (20-30 g). These cheeses were placed in a 15% brine solution (1 L) containing 0.1% $CaCl_2$ adjusted to pH 3.5 with citric acid for 2 h at room temperature. The brined cheeses were then vacuum sealed and stored at 5°C and 15°C.

Two batches of each raw and pasteurised milk Feta were prepared, each containing a different combination of strain of *E. coli* or species of *Listeria*. Samples were taken for microbiological and chemical analyses, as described below, at various times during the cheese making and maturation process.

Double-cream Brie Cheese

Both pasteurised and raw milk at 38°C were combined with long life cream (14.5 ml/L), $CaCl_2$ (100 mg/L), Type B and E cultures (2 mg/L in a ratio of 1:1), white mould spore powder (*Penicillium candidum*; 1 mg/L) and target bacteria (a mixture of one strain of *E. coli* and one species of *Listeria* in a ratio of 1:1; approximately 10^{4-5} CFU/ml). The milk mixture was kept for 1 h before the addition of "vegetarian" rennet (40 IMCU/L).

After a further 30 minutes, the resulting coagulum was cut in cubes and allowed to settle for 30 min. Thereafter, the curd was gently turned over and allowed to rest for 20 min. This turning step was then repeated twice before most of the whey was drained off. The remaining curd was transferred to a plastic hoop and turned after 10 min, 40 min, 2 h and 5 h to achieve even whey drainage and even shape. The curd was then kept at 27°C for 20-22 h.

Cheese was removed from the hoop and divided into four equal pieces of cheese (approximately 150 g each). All cheeses were placed in a 20% brine solution (2 L) for 40 min at room temperature. Cheeses were then transferred onto a rack and left for ripening under high humidity conditions at 15°C for eight days, and finally loosely wrapped and stored at the same temperature for another two weeks.

Two batches of each raw and pasteurised milk double-cream Brie were prepared, each containing a different combination of strain of *E. coli* or species of *Listeria*. Samples were taken for microbiological and chemical analyses, as described below, at various times during the cheese making and maturation process.

Wensleydale Cheese

When the milk reached 32°C, type A starter culture (2mg), rennet and challenge bacteria were added to 10L or either raw or pasteurised milk. The rennet was added to the pasteurized milk over

1.5 minutes while the milk was constantly mixed. The milk was held at 32°C for ~ 1 h to let the curd form.

After curd formation, the curd was cut vertically followed by another set of vertical cuts at 90° to the first, resulting in cubes of approximately 2-3 cm. The curd was then cut again at a 45° angle.

After cutting the curd was gently heated to 38°C for one hour to aid further whey expulsion. During this step the curd and whey were continuously stirred and any large pieces of curd were cut into pieces no larger than 12 mm. Curds were left to settle for 30 minutes at 38°C, and then transferred into plastic hoops (two per milk type). Whey was removed, sampled and aseptically discarded. The hoops were then incubated at 38-40°C for 60 minutes to allow the curds to settle and knit together. After settling, the curds were inverted in the hoops and incubated for a further 60 minutes at 38-40°C.

The hooped curds were transferred into containers, with the base of the hoop raised from the bottom of the containers to facilitate whey expulsion. These containers were covered with aluminium foil and incubated at 15°C for 15 - 20 hours. Each cheese was then removed from its hoop and placed in 6L of brine (saturated salt solution consisting of 350g of NaCl per L of distilled water) for two hours at room temperature.

After drying for four days at 15°C, each cheese was cut into wedges (~50g) yielding a minimum of 14 pieces of cheese per milk type (raw or pasteurized). Each piece was into molten wax (Cheeselinks http://cheeselinks.com.au/) for a 3-5 seconds, so that half to two-thirds of the wedge of cheese was covered with wax. The piece was then set aside to dry. Once dry (~5 minutes) the cheese wedge was inverted and dipped in the wax again ensuring that this dipping covered the initial dip line. This process was then repeated so that the wedge of cheese was coated in two layers of wax. The waxed pieces were then left for an hour at room temperature to set. Once set the cheese wedges were placed in containers with a loose lid covering them in a fan-forced incubator set to 15°C for maturation for up to 12 weeks.

Microbiological Analyses

Milk and cheese samples were aseptically collected during the cheese manufacturing process. To determine numbers of *E. coli* and *Listeria* spp. in these samples, Australian Standard (AS 1766.3.15-1994) was employed with some modifications. Briefly, milk (0.1 ml) or cheese (5 g) sample was diluted 1:10 with diluent containing 1% sodium citrate (Merck 1. 06448. 0500; 20g per 1000mL) and 0.04% peptone water as per Australian Standard (AS 5013.11.5). Only cheese samples were stomached for 5 min in a Colworth stomacher 400 (A.J. Seward Ltd, UK). Both milk and cheese mixtures were serially diluted in sterile 0.1% peptone water. The appropriate dilutions of each sample were surface-plated on selective agars (EMB for E. coli, PALCAM for Listeria; Oxoid) using an Autoplate 4000 spiral plater (Spiral Biotech, Norwood, MA). All EMB and PALCAM plates were then incubated for 24 h and 48 h at 37 ºC, respectively before presumptive colonies on the plates were counted from the appropriate dilutions. The number of CFU per ml (or g) in the original sample was then calculated following the formulae described in the manufacturer's instruction of the spiral plater.

Physico-chemical Analyses

All milk and cheese samples were analysed for their pH and water activity. In addition, a number of samples were selected based on their changes in pH level to determine their lactic acid concentration. In all cheese styles, except double-cream Brie cheese, the interior and the surface of the cheese were analysed separately.

pH measurement

The pH of milk samples was measured directly using a calibrated digital pH meter and probe. For cheese samples, 2 g of each sample was combined with 4 ml of non-sterile distilled water. The mixture was stomached (Colworth Stomacher 400, A.J. Seward Ltd) for 5 min. Thereafter, the pH of the homogenised sample was measured using a calibrated digital pH meter and probe (Orion 250A pH meter).

Water Activity Measurement

The water activity of each sample was measured using an Aqualab CX2 dew-point instrument (Decagon Devices, Pullman, WA, USA). Cheese samples (~2g) were chopped into small pieces to increase surface area and speed the rate of equilibration of humidity. For liquid samples, 2 – 3 mL of sample were used.

Determination of L-lactic Acid Content

Milk (5 g) or cheese (1 g) sample was weighed out and transferred to a 50 ml falcon tube containing 30 ml of distilled water. This mixture was heated at 70°C with occasional shaking for 30 min and then mixed with approximately 20 ml of distilled water. After holding on ice for 30 min, the mixture was filtered. An appropriate volume of sample was assayed for L-lactic acid concentration using a commercial test kit, (K-LATE, Megazyme International Ireland Limited, Ireland), according to the manufacturer's instructions. Optical density was measured at 340 nm (Spectrostar Nano, BMG, Labtech).

APPENDIX 9. Analysis of Growth/No Growth Models

This Appendix tabulates details of time-dependent physico-chemical changes in the various cheeses produced for the challenge trials described in Sections 4 and 5. For some cheeses (including all Wensleydale and some double-cream Brie), lactic acid data were not available. As discussed in Section 5, lactic acid concentration is often a key determinant of growth potential. Accordingly, where those data were not available no predictions from the models were made.

In the Tables, the following models are evaluated:

vii) *Listeria moncytogenes* growth rate model of Mejlholm and Dalgaard (2007)
viii) *Listeria moncytogenes* growth probability model of Mejlholm and Dalgaard (2007)
ix) Unpublished growth rate model for *L. monocytogenes* developed at University of Tasmania
x) *Escherichia coli* growth rate model of Ross *et al.* (2003)
xi) Updated probability of growth model for *L. monocytogenes strains* Scott A and L5 developed at University of Tasmania based on the models of Tienungoon *et al.* (2000)
xii) *Escherichia coli* probability of growth model of Presser *et al*. (1998)

The 'probability of growth' models were also compared to predictions of growth/no growth based simply on the limits to growth of *L. monocytogenes* and *E. coli* presented in Table 5.8. No growth was predicted if the pH, a_w or undissociated lactic acid concentration in the cheese is beyond the limit for growth of the respective organisms. This approach was taken to assess whether useful additional discrimination was achieved using the more complex growth/no growth models because, for much of the data, the physico-chemical conditions in the cheese present either very high probability of growth or very low probability of growth. The most discriminating data to assess the performance of the models is the point of transition from growth permissive to non-growth permissive and, in essence, relates to only 2 to 3 observations per trial, with the exception of the double-cream Brie trials in which there are two transitions between growth permissive and non-growth permissive conditions, *i.e.*, one associated with curd formation and the other associated with the increase in pH due to fungal catabolism of proteins during maturation.

Because not all physico-chemical data were recorded at each sample time, where specific data were not available, data from the previous sample was used to enable model predictions. This mainly relates to measurement of lactic acid concentration. In cases where this occurred, the values are shown in grey text, not black. In general, these 'default' values are identical to the last measured observation except for the undissociated lactic acid concentrations because pH values were determined at every sampling time, while lactic acid levels were not. Accordingly, the undissociated lactic acid levels based on the previous result can change due to a different pH in the 'current' result.

In the tables of data, areas in dark grey shading indicate times when consistent growth, since the previous viable count determination, was not observed in the cheese. Dark grey shading is also used to indicate *predictions* of no growth from the various models. For the UTas and Presser *et al*. (1998) models, values of $P < 0.25$ are interpreted as no growth, and predictions of $P>0.75$ as growth. Light grey shading is used where 'marginal' growth is predicted. In the case of the UTas and Presser *et al.*

models, marginal growth is considered to be when the predicted probability of growth is between 0.25 and 0.75. For the Mejlholm and Dalgaard (2007) model, a value of Psi ≥ 1 is interpreted as no growth, while values in the range 0.5 to 1 are considered as marginal. Psi values below 0.5 have been interpreted as growth permissive. Accordingly, in this scheme where shading differs between the observed data cells and the growth/no growth prediction cells, a disagreement is indicated.

In the case of the simple growth/no growth prediction based on individual limits, pale orange shading is used to indicate disagreement with predictions from the more complex growth/no growth models.

				Water content (ex Table	^											
				0.360					*L. mono*	Psi	*L. mono*	*E. coli*				
								Inferred	Gen Time (min)	(L. mono)	Gen Time (min)	Gen Time (min)	P (L. mono)	P (E. coli)	*L. mono*	*E. coli*
Time (h)	Temperature	pH	Aw	undiss Lactic Acid	Lactic Acid	Lactic Acid	LA	Salt in Water	Mejholm and	Mejholm and	(Utas, unpub.)	(Ross et al., 2003)	(Utas, unpub)	Presser	simple	simple
	(°C)			(mM)	(g/100g cheese)	(g/100g water)	(mM aqueous)	(%)	Dalgaard (2007)	Dalgaard (2007)				et al. (1998)	minima	minima
Cheddar Raw (M23, L. innocua)																
0.00	32	6.71	0.994	0.009	0.02	0.06	6.17	1.11							growth	growth
1.08	32	6.71	0.996	0.009	0.02	0.06	6.17	0.75							growth	growth
1.75	38	6.60	0.997	0.011	0.02	0.06	6.17	0.56							growth	growth
3.27	38	6.27	0.996	0.024	0.02	0.06	6.17	0.75							growth	growth
4.77	38	6.67	0.994	0.010	0.02	0.06	6.17	1.11	26	0.11	41	17	0.999	1.000	growth	growth
5.92	27	5.55	0.967	3.149	0.51	1.42	157.37	5.57	616	0.72	450	159	0.926	0.924	growth	growth
25.50	27	5.36	0.966	8.891	0.94	2.61	290.06	5.72	ng	1.28	ng	955	0.076	0.000	ng	growth
50.50	27	5.38	0.952	8.503	0.94	2.61	290.06	7.75	ng	1.15	ng	9653	0.069	0.000	ng	growth
99.50	10	5.48	0.949	6.144	0.85	2.36	262.29	8.17	ng	0.84	ng	ng	0.370	0.000	ng	ng
Cheddar Pasteurised (M23, L. innocua)																
0.00	32	6.72	0.994	0.002	0.01	0.01	1.58	1.11							growth	growth
1.08	32	6.74	0.993	0.002	0.01	0.01	1.58	1.38							growth	growth
1.75	38	6.47	0.996	0.004	0.01	0.01	1.58	0.84							growth	growth
3.27	38	6.12	0.997	0.003	0.00	0.00	0.49	0.66	49	0.00	41	61	0.996	1.000	growth	growth
4.77	38	5.50	0.992	0.011	0.00	0.00	0.49	1.47	66	0.02	95	44	0.991	1.000	growth	growth
5.92	27	5.41	0.952	5.108	0.60	1.68	186.36	7.75	ng	0.53	ng	3058	0.592	0.000	ng	growth
25.50	27	5.26	0.970	11.848	1.00	2.79	309.45	5.19	ng	1.79	ng	ng	0.003	0.000	ng	ng
50.50	27	5.34	0.958	9.919	1.00	2.79	309.45	6.97	ng	1.51	ng	6237	0.021	0.000	ng	growth
99.50	10	5.29	0.954	11.457	1.04	2.88	319.83	7.47	ng	1.21	ng	ng	0.003	0.000	ng	ng

Table A9.1. Physico-chemical data for Cheddar cheeses and model predictions

Time (h)	Temperature (°C)	pH	Aw	undiss Lactic Acid (mM)	Lactic Acid (g/100g cheese)	Lactic Acid (g/100g water)	LA (mM aqueous)	Inferred Salt in Water (%)	*L. mono* Gen Time (min) Mejholm and Dalgaard (2007)	Psi (L. mono) Mejholm and Dalgaard (2007)	*L. mono* Gen Time (min) (Utas, unpub.)	*E. coli* Gen Time (min) (Ross et al., 2003)	P (L. mono) (Utas, unpub)	P (E. coli) Presser et al. (1998)	*L. mono* simple minima	*E. coli* simple minima
Cheddar Raw (R31, L.mono)																
0.00	32	6.71	0.991	0.002	0.00	0.01	1.38	1.74							growth	growth
1.08	32	6.66	0.991	0.002	0.00	0.01	1.38	1.74							growth	growth
2.00	38	6.56	0.992	0.003	0.00	0.01	1.38	1.56							growth	growth
3.42	38	6.31	0.996	0.031	0.03	0.08	8.90	0.84							growth	growth
4.92	38	5.38	0.994	0.261	0.03	0.08	8.90	1.11	44	0.20	67	18	0.989	1.000	growth	growth
5.92	27	4.90	0.970	11.114	0.43	1.20	132.98	5.11	210	>2	ng	ng	0.004	0.000	ng	ng
24.50	27	5.24	0.976	7.775	0.63	1.75	194.27	4.17	ng	1.11	ng	298	0.221	0.173	ng	growth
49.50	27	5.20	0.952	8.492	0.63	1.75	194.27	7.82	ng	1.39	ng	8615	0.057	0.000	ng	growth
98.50	10	5.21	0.931	8.307	0.63	1.75	194.27	10.56	ng	2.60	ng	ng	0.017	0.000	ng	ng
Cheddar Pasteurised (R31, L. mono)																
0.00	32	6.74	0.990	0.012	0.03	0.08	9.29	1.83							growth	growth
1.08	32	6.71	0.993	0.013	0.03	0.08	9.29	1.38							growth	growth
2.00	38	6.54	0.994	0.019	0.03	0.08	9.29	1.20							growth	growth
3.42	38	6.32	0.996	0.032	0.03	0.08	9.39	0.84	26	0.12	41	18	1.000	1.000	growth	growth
4.92	38	5.30	0.993	0.329	0.03	0.08	9.39	1.38	53	0.24	77	19	1.000	1.000	growth	growth
5.92	27	5.03	0.962	7.638	0.39	1.09	120.62	6.39	ng	1.59	ng	600	0.130	0.003	ng	growth
24.50	27	5.19	0.977	9.233	0.67	1.86	206.63	4.09	ng	1.40	ng	660	0.058	0.002	ng	growth
49.50	27	5.15	0.942	10.080	0.67	1.86	206.63	9.12	ng	2.01	ng	ng	0.007	0.000	ng	ng
98.50	10	5.21	0.944	8.835	0.67	1.86	206.63	8.92	ng	2.06	ng	ng	0.029	0.000	ng	ng

Table A9.1 (cont.). Physico-chemical data for Cheddar cheeses and model predictions

Time (h)	Temperature (°C)	pH	Aw	undiss Lactic Acid (mM)	Lactic Acid (g/100g cheese)	Lactic Acid (g/100g water)	LA (mM aqueous)	Inferred Salt in Water (%)	*L. mono* Gen Time (min) Mejholm and Dalgaard (2007)	Psi (L. mono) Mejholm and Dalgaard (2007)	*L. mono* Gen Time (min) (Utas, unpub.)	*E. coli* Gen Time (min) (Ross et al., 2003)	P (L. mono) (Utas, unpub)	P (E. coli) Presser et al. (1998)	*L. mono* simple minima	*E. coli* simple minima
Gouda Raw (M23, L. innocua)					0.42											
Raw milk								Inferred	Gen Time	P (Lm)	Gen Time	Gen Time	P(Lm)	P (E. coli)		
Time (h)	**Temperature (°C)**	**pH**	**Aw**	**undiss LA (mM)**	**LA (g/100g)**	**LA (g/100g WATER)**	**LA (mM in water)**	**Salt in Water (%)**	**(DalgaarD)**	**Mejholm Dalgaard**	**(UTas)**	**(UTas)**	**Tienungoon**	**Presser et al**		
0.00	32	6.73	0.995	0.000	0.00	0.00	0.34	0.93							growth	growth
1.00	32	6.74	0.994	0.000	0.00	0.00	0.34	1.11							growth	growth
1.75	32	6.68	0.991	0.001	0.00	0.00	0.34	1.65							growth	growth
2.75	38	6.58	0.993	0.001	0.00	0.00	0.34	1.29							growth	growth
3.67	38	6.39	0.994	0.045	0.06	0.14	15.25	1.11	40	0.16	41	17	0.998	1.000	growth	growth
24.17	22	5.27	0.989	8.219	0.83	1.98	219.48	2.00	ng	1.16	ng	393	0.180	0.421	ng	growth
46.92	22	5.31	0.921	7.521	0.83	1.98	219.48	11.81	ng	>1.2	ng	ng	0.000	0.000	ng	ng
99.00	15	5.26	0.934	7.900	0.78	1.86	206.35	10.17	ng	1.63	ng	ng	0.014	0.000	ng	ng
Gouda Pasteurised (M23, L. innocua)																
0.00	32	6.76	0.991	0.001	0.00	0.01	0.68	1.65							growth	growth
1.00	32	6.77	0.998	0.001	0.00	0.01	0.68	0.38							growth	growth
1.75	32	6.70	0.989	0.001	0.00	0.01	0.68	2.00							growth	growth
2.75	38	6.55	0.993	0.001	0.00	0.01	0.68	1.29	26	0.11	42	17	0.999	1.000	growth	growth
3.67	38	6.28	0.997	0.053	0.05	0.13	13.98	0.56	26	0.12	41	16	0.997	1.000	growth	growth
24.17	22	5.22	0.991	9.145	0.83	1.97	218.64	1.65	ng	1.34	ng	660	0.073	0.034	ng	growth
46.92	22	5.27	0.944	8.187	0.83	1.97	218.64	8.85	ng	1.39	ng	ng	0.045	0.000	ng	ng
99.00	15	5.27	0.944	8.362	0.84	2.01	223.30	8.85	ng	1.55	ng	ng	0.019	0.000	ng	ng

Table A9.2. Physico-chemical data for Gouda cheeses and model predictions

Time (h)	Temperature (°C)	pH	Aw	undiss Lactic Acid (mM)	Lactic Acid (g/100g cheese)	Lactic Acid (g/100g water)	LA (mM aqueous)	Inferred Salt in Water (%)	*L. mono* Gen Time (min) Mejholm and Dalgaard (2007)	Psi (L. mono) Mejholm and Dalgaard (2007)	*L. mono* Gen Time (min) (Utas, unpub.)	*E. coli* Gen Time (min) (Ross et al., 2003)	P (L. mono) (Utas, unpub)	P (E. coli) Presser et al. (1998)	*L. mono* simple minima	*E. coli* simple minima
Gouda Raw (R31, L.mono)																
0.00	32	6.66	0.997	0.001	0.00	0.01	0.85	0.56							growth	growth
1.00	32	6.70	0.995	0.001	0.00	0.01	0.85	0.93							growth	growth
1.75	32	6.63	0.993	0.001	0.00	0.01	0.85	1.38							growth	growth
2.83	38	6.45	0.997	0.002	0.00	0.01	0.85	0.56	25	0.11	40	16	0.998	1.000	growth	growth
3.75	38	6.35	0.998	0.003	0.00	0.01	0.85	0.38	25	0.11	40	16	0.998	1.000	growth	growth
24.25	22	5.17	0.991	8.824	0.71	1.70	188.98	1.65	ng	1.33	ng	505	0.093	0.138	ng	growth
46.75	22	5.28	0.942	6.922	0.71	1.70	188.98	9.12	ng	1.11	ng	ng	0.112	0.000	ng	ng
100.83	15	5.32	0.939	7.341	0.83	1.97	219.06	9.52	ng	1.28	ng	ng	0.035	0.000	ng	ng
Gouda Pasteurised (R31, L. mono)																
0.00	32	6.77	0.992	0.002	0.01	0.02	2.03	1.56							growth	growth
1.00	32	6.72	0.993	0.003	0.01	0.02	2.03	1.38							growth	growth
1.75	32	6.60	0.997	0.004	0.01	0.02	2.03	0.66							growth	growth
2.83	38	6.46	0.999	0.005	0.01	0.02	2.03	0.28	25	0.11	39	16	0.998	1.000	growth	growth
3.75	38	6.36	0.998	0.031	0.04	0.09	9.75	0.47	25	0.12	40	16	0.998	1.000	growth	growth
24.25	22	5.26	0.990	6.294	0.62	1.48	164.40	1.92	ng	0.86	ng	193	0.568	0.984	ng	growth
46.75	22	5.32	0.919	14.373	1.62	3.86	428.89	12.05	ng	>8	ng	ng	0.000	0.000	ng	ng
100.83	15	5.33	0.934	5.569	0.64	1.53	169.91	10.24	ng	0.61	ng	ng	0.086	0.000	ng	ng

Table A9.2 (cont). Physico-chemical data for Gouda cheeses and model predictions

Time (h)	Temperature (°C)	pH	Aw	undiss Lactic Acid (mM)	Lactic Acid (g/100g cheese)	Lactic Acid (g/100g water)	LA (mM aqueous)	Inferred Salt in Water (%)	*L. mono* Gen Time (min) Mejholm and Dalgaard (2007)	Psi (L. mono) Mejholm and Dalgaard (2007)	*L. mono* Gen Time (min) (Utas, unpub.)	*E. coli* Gen Time (min) (Ross et al., 2003)	P (L. mono) (Utas, unpub)	P (E. coli) Presser et al. (1998)	*L. mono* simple minima	*E. coli* simple minima
Feta Cheese					Water content (Table 1)											
Feta Raw (M23, L. innocua)					0.54											
0.00	34.00	6.79	0.997	0.000	0.00	0.00	0.26	0.56							growth	growth
1.00	34.00	6.75	0.997	0.000	0.00	0.00	0.26	0.66							growth	growth
2.08	34.00	6.70	0.998	0.000	0.00	0.00	0.26	0.47							growth	growth
4.08	34.00	6.47	0.996	0.010	0.02	0.04	3.95	0.84	31	0.05	44	20	0.543	1.000	growth	growth
5.92	34.00	5.84	0.997	0.041	0.02	0.04	3.95	0.66	35	0.06	47	20	0.376	1.000	growth	growth
6.92	34.00	5.55	0.997	1.998	0.49	0.90	99.86	0.66	85	0.44	100	27	0.145	1.000	growth	growth
24.08	20.00	5.09	0.990	11.454	1.00	1.85	205.97	1.83	ng	0.68	ng	ng	0.003	0.000	ng	ng
26.25	20.00	5.27	0.956	7.713	1.00	1.85	205.97	7.26	ng	1.17	ng	ng	0.096	0.000	ng	growth
52.58	20.00	5.22	0.961	8.780	1.02	1.89	209.93	6.46	ng	1.34	ng	ng	0.043	0.000	ng	growth
Feta Pasteurised (M23, L. innocua)																
0.00	34.00	6.82	0.996	0.000	0.00	0.00	0.33	0.75							growth	growth
1.00	34.00	6.76	0.995	0.000	0.00	0.00	0.33	0.93							growth	growth
2.08	34.00	6.7	0.997	0.000	0.00	0.00	0.33	0.56							growth	growth
4.08	34.00	6.25	0.995	0.044	0.05	0.10	10.88	1.02	33	0.06	44	20	0.463	1.000	growth	growth
5.92	34.00	5.8	0.995	0.123	0.05	0.10	10.88	0.93	37	0.08	50	21	0.355	1.000	growth	growth
6.92	34.00	5.6	0.995	1.578	0.43	0.80	88.32	0.93	68	0.35	85	27	0.182	1.000	growth	growth
24.08	20.00	5.12	0.988	10.506	0.98	1.82	201.69	2.18	ng	1.76	ng	ng	0.011	0.000	ng	ng
26.25	20.00	5.31	0.954	6.911	0.98	1.82	201.69	7.47	ng	1.01	ng	ng	0.174	0.000	ng	growth
52.58	20.00	5.25	0.950	4.373	0.54	1.01	111.72	8.10	ng	>2	ng	ng	0.548	0.000	growth	ng

Table A9.3 Physico-chemical data for Feta cheeses and model predictions

Time (h)	Temperature (°C)	pH	Aw	undiss Lactic Acid (mM)	Lactic Acid (g/100g cheese)	Lactic Acid (g/100g water)	LA (mM aqueous)	Inferred Salt in Water (%)	*L. mono* Gen Time (min) Mejholm and Dalgaard (2007)	Psi (L. mono) Mejholm and Dalgaard (2007)	*L. mono* Gen Time (min) (Utas, unpub.)	*E. coli* Gen Time (min) (Ross et al., 2003)	P (L. mono) (Utas, unpub)	P (E. coli) Presser et al. (1998)	*L. mono* simple minima	*E. coli* simple minima
Feta Raw (R31, L. monocytogenes)																
0.00	34.00	6.75	0.997	0.004	0.02	0.03	3.43	0.66							growth	growth
1.00	34.00	6.75	0.996	0.004	0.02	0.03	3.43	0.84							growth	growth
2.17	34.00	6.59	0.996	0.006	0.02	0.03	3.43	0.75							growth	growth
4.08	34.00	6.2	0.995	0.070	0.08	0.14	15.49	0.93	33	0.07	45	21	0.447	1.000	growth	growth
5.92	34.00	5.8	0.995	0.176	0.08	0.14	15.49	1.02	38	0.09	51	21	0.350	1.000	growth	growth
6.92	34.00	5.52	0.996	1.094	0.25	0.46	51.08	0.84	59	0.26	75	24	0.214	1.000	growth	growth
24.08	20.00	4.83	0.994	14.448	0.73	1.34	149.29	1.20	ng	>3	184	ng	0.000	0.000	ng	ng
26.17	20.00	5.88	0.952	1.412	0.73	1.34	149.29	7.75	360	0.39	465	3500	0.952	0.004	growth	growth
47.58	20.00	5.68	0.944	2.501	0.82	1.51	167.74	8.85	1000	0.89	1110	ng	0.842	0.000	growth	ng
Feta Pasteurised (R31, L. monocytogenes)																
0.00	34.00	6.79	0.993	0.000	0.00	0.00	0.26	1.29							growth	growth
1.00	34.00	6.67	0.994	0.000	0.00	0.00	0.26	1.20							growth	growth
2.17	34.00	6.58	0.993	0.001	0.00	0.00	0.26	1.29							growth	growth
4.08	34.00	6.3	0.995	0.019	0.03	0.05	5.27	0.93	32	0.06	44	21	0.479	1.000	growth	growth
5.92	34.00	5.4	0.993	0.148	0.03	0.05	5.27	1.29	52	0.11	68	22	0.263	1.000	growth	growth
6.92	34.00	5.33	0.995	2.754	0.41	0.76	84.04	0.93	200	0.77	185	31	0.064	1.000	growth	growth
24.08	20.00	4.9	0.995	16.912	0.98	1.82	202.35	1.02	ng	>4	ng	ng	0.000	0.000	ng	ng
26.17	20.00	5.8	0.938	2.297	0.98	1.82	202.35	9.65	1150	0.95	1430	ng	0.814	0.000	growth	ng
47.58	20.00	5.64	0.918	2.512	0.75	1.39	153.90	12.18	ng	>7	ng	ng	0.000	0.000	ng	ng

Table A9.3 (cont). Physico-chemical data for Feta cheeses and model predictions

Time (h)	Temperature (°C)	pH	Aw	undiss Lactic Acid (mM)	Lactic Acid (g/100g cheese)	Lactic Acid (g/100g water)	LA (mM aqueous)	Inferred Salt in Water (%)	*L. mono* Gen Time (min) Mejholm and Dalgaard (2007)	Psi (L. mono) Mejholm and Dalgaard (2007)	*L. mono* Gen Time (min) (Utas, unpub.)	*E. coli* Gen Time (min) (Ross et al., 2003)	P (L. mono) (Utas, unpub)	P (E. coli) Presser et al. (1998)	*L. mono* simple minima	*E. coli* simple minima
			Water content (Table 1)													
			0.490													
double cream Brie																
Raw milk																
0.00	38	6.57	0.995	0.002	0.0035244	0.01	0.80	1.02	26	0.11	41	17	0.999	1.000	growth	growth
1.08	38	6.59	0.992	0.001	0.0035244	0.01	0.80	1.47	27	0.12	42	18	0.999	1.000	growth	growth
1.58	38	6.58	0.994	0.002	0.0035244	0.01	0.80	1.11	26	0.11	41	17	0.999	1.000	growth	growth
3.33	38	6.59	0.995	0.001	0.0035244	0.01	0.80	1.02	26	0.11	41	17	0.999	1.000	growth	growth
5.50	38	5.99	0.996	0.596	0.357246	0.73	80.99	0.84	81	0.23	78	22	0.983	1.000	growth	growth
23.08	27	5.17	0.993	9.191	0.868284	1.77	196.85	1.38	ng	1.47	ng	388	0.083	0.072	ng	growth
23.82	27	5.16	0.972	9.395	0.868284	1.77	196.85	4.80	ng	1.66	ng	926	0.042	0.000	ng	growth
95.83	15	5.19	0.975	9.769	0.964404	1.97	218.64	4.41	ng	1.41	ng	5697	0.012	0.000	ng	growth
266.25	15	5.66	0.979	3.411	0.96	1.97	218.64	3.77	218	0.11	1301	448	0.858	0.983	growth	growth
432.75	15	7.14	0.968	0.003	0.025632	0.05	5.81	5.49	212	0.10	295	390	1.000	0.990	growth	growth
622.83	15	7.5	0.966	0.001	0.025632	0.05	5.81	5.77	220	0.11	309	452	1.000	0.978	growth	growth
771.67	15	7.59	0.966	0.001	0.025632	0.05	5.81	5.76	220	0.11	308	457	1.000	0.978	growth	growth
1009.00	15	7.9	0.963	0.001	0.030438	0.06	6.90	6.17	234	0.12	332	605	1.000	0.929	growth	growth
Pasteurised milk																
0.00	38	6.56	0.992	0.001	0.0025632	0.01	0.58	1.56	27	0.11	43	18	0.999	1.000	growth	growth
1.08	38	6.58	0.993	0.001	0.0025632	0.01	0.58	1.38	26	0.11	42	18	0.999	1.000	growth	growth
1.58	38	6.59	0.994	0.001	0.0025632	0.01	0.58	1.11	26	0.11	41	17	0.999	1.000	growth	growth
3.33	38	6.6	0.994	0.001	0.0025632	0.01	0.58	1.20	26	0.11	41	17	0.999	1.000	growth	growth
5.50	27	5.92	0.994	0.605	0.309186	0.63	70.09	1.11	63	0.09	78	40	0.992	1.000	growth	growth
23.08	27	5.19	0.993	9.348	0.922752	1.88	209.19	1.29	ng	1.39	ng	447	0.074	0.034	ng	growth
23.82	27	5.16	0.968	9.984	0.922752	1.88	209.19	5.42	ng	1.60	ng	2670	0.021	0.000	ng	growth
95.83	15	5.22	0.970	9.190	0.96921	1.98	219.73	5.11	ng	1.30	ng	3819	0.021	0.000	ng	growth
266.25	15	6.85	0.971	0.225	0.96921	1.98	219.73	5.03	212	0.14	330	428	0.999	0.988	growth	growth
432.75	15	7.47	0.964	0.003	0.052866	0.11	11.99	6.02	230	0.11	325	523	1.000	0.975	growth	growth
622.83	15	7.37	0.965	0.004	0.052866	0.11	11.99	5.91	223	0.11	318	481	1.000	0.967	growth	growth
771.67	15	7.56	0.963	0.002	0.052866	0.11	11.99	6.17	235	0.12	334	571	1.000	0.927	growth	growth
1009.00	15	7.91	0.962	0.000	0.022428	0.05	5.08	6.31	240	0.12	341	661	1.000	0.892	growth	growth

Table A9.4 Physico-chemical data for Brie cheeses and model predictions

APPENDIX 10. Details of Unpublished Growth/No Growth Models

i) Unpublished growth rate model for *L. monocytogenes* developed at University of Tasmania based on data of Tienungoon (1998).

$$\text{Generation time (h)} = 1/(0.02349 \times (a_w-0.925)*(T-0.60)^2 \times (1-e^{(0.129*(T-50.95))})^2*(1-10^{(4.94-pH)}) \times (1-LA/(4.55*(1+10^{(pH-3.86)}))) \times (1-LA/(1821.91 \times (1+10^{(3.86-pH)})))$$

Where a_w = water activity

T = temperature (°C)

pH has its usual meaning

LA = total lactic acid concentration (mM)

ii) Updated probability-of-growth model for *L. monocytogenes strains* Scott A and L5 developed at University of Tasmania based on the original models of Tienungoon *et al.* (2000).

$$P_{(growth)} = e^y/(1 + e^y)$$

Where y = max (**A, B**) where:

$$\mathbf{A} = -25.36+44.12 \times ln(T+1.623)-7.0222 \times (ln(T+1.623))^2+ 10257 \times ln(1-e^{(0.536*(T-48))})+8.951 \times ln(a_w-0.9152)+291.8* ln (1-10^{(3.35-pH)}) +704.1 \times (ln(1-10^{(3.35-pH)}))^2+58.12 \times ln(1-(LA/(25*(1+10^{(pH-3.84)}))))$$

$$\mathbf{B} = -6.023+19 \times ln(T-0.416)- 3.049 \times (ln(T-0.416))^2+ 7514 \times ln(1-e^{(0.536*(T-48))})+4.635 \times ln(a_w-0.9142)+141* ln (1-10^{(3.35-pH)}) +240.2 \times (ln(1-10^{(3.35-pH)}))^2+31.98 \times ln(1-(LA/(23.68*(1+10^{(pH-3.84)}))))$$

Where a_w = water activity

T = temperature (°C)

pH has its usual meaning

LA = total lactic acid concentration (mM)

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