Ministry for Primary Industries
Manatū Ahu Matua

Effect of Microbial Interactions on Pathogen Growth and Survival during Fermentation of Raw Milk – Final Report

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This study provides an understanding of chemical changes in milk during the fermentation process and the effect of these changes on pathogen dynamics in raw and pasteurised milk. While the knowledge gained significantly contributes to a scientific background for cheese-making, this particular work examines the fermentation process in liquid milk and does not follow any specific cheese-making protocols.

This study confirmed the previously reported observation (Schvartzman et al, 2011) that achievement of the desired lactic acid concentration, and hence pH, is delayed during fermentation of raw milk compared to fermentation of matched pasteurised milk. Moreover, it was shown that the naturally-occurring flora in raw milk does not inhibit starter culture survival or growth, but rather appears to affect its biochemical activity.

The study also showed that the rates of pH decrease during fermentation vary between different milks even when the same starter cultures, and same inoculum level, are used. This observation highlights the critical importance of monitoring pH change during the cheese-making process.

In addition, the naturally-occurring flora in raw milk was not observed to inhibit pathogen survival.

Lastly, *Staphylococcus aureus* was observed to grow in milk both when added in challenge experiments and when present as a natural contaminant. When added, growth was similar in the presence and absence of starter culture, albeit after a long lag period. This highlights the need to ensure that the animals are free of mastitis and the milk is of the highest quality in terms of *S. aureus* when used for raw milk cheese production.

Client Report – FBP 12509

Effect of Microbial Interactions on Pathogen Growth and Survival during Fermentation of Raw Milk – Final Report

Ministry of Primary Industries

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Abstract

This report presents data from two challenge trials carried out as part of a project to investigate whether the growth and ultimately the survival of two foodborne pathogens, *Listeria monocytogenes* and *Staphylococcus aureus,* is inhibited during the early stages of milk fermentation by starter cultures used in the cheese industry. Data can be used to populate models that describe the effect of chemical changes during fermentation on pathogen growth dynamics. Further, this project will determine whether the presence of naturally occurring raw microflora influenced survival and growth of *L. monocytogenes* and *S. aureus* during fermentation.

These challenge trials were performed at two levels of inoculum; high inoculum bacterial cocktails composed of six milk-isolates of *L. monocytogenes* and *S. aureus;* and low inoculum suspensions comprised of individual isolates. Two representative cheese starter cultures from Chr. Hansen A/S were used to simulate the initial stages of milk fermentation which results in the production of lactic acid and the reduction of pH that occurs during commercial cheese manufacture. Milk samples were obtained from the same supplier for use in these challenge trials. A pasteurised milk sample was prepared from the same batch of raw milk supplied for testing to provide a matched pair of milk samples per trial.

Data presented show that neither *L. monocytogenes* nor *S. aureus* was eliminated during fermentation of either raw or pasteurised milk, which was carried out as per manufacturers' instructions. After an increased lag period, *S. aureus* numbers increased in the presence of the starter cultures and achieved similar growth rates to those observed with no starter culture present. No change in *L. monocytogenes* numbers were observed in challenge trials carried out in the presence of either starter culture nor in the pathogen only control milk samples. In the presence of both cheese starter cultures, pH was shown to slowly decrease over the course of the fermentation for both raw and pasteurised milk. In general, both trials showed noticeably higher pH values for raw milk than for pasteurised milk in the latter phase of the fermentation. This difference was most noticeable for FD-DVS pHageControl™ R-704. In general, lactic acid concentration was observed to increase over time, in line with observed decreases in pH. In conclusion, under the test conditions used here, there was no significant reduction in either *Listeria* or *Staphylococcus* numbers during fermentation of either raw or pasteurised milk.

Contents

1. Introduction

Microbial safety is an implicit expectation of food consumers. Manufacturers and producers strive to fulfil this expectation and apply HACCP measures to ensure that this is the case. However, for these measures to be effective, scientific evidence must be to prove efficacy against food borne pathogens of concern. Currently, there is a move to minimise food processing and limit the use of preservatives and the like; even those that have historically been used such as salt and sugar. Milk and milk products are traditionally heat-treated or pasteurised to remove pathogenic bacteria, particularly *Mycobacterium tuberculosis.* Pasteurisation is a cost effective method of pathogen removal from complex biological product such as milk without adversely interfering with product quality. However, there is growing consumer support for the right to consume raw milk and milk products such as cheeses that are manufactured from raw milk as they perceive that the benefits outweigh the risks (Oliver et al, 2009).

In New Zealand regulations permitting the consumption of some raw milk products have been in place since 2009. Under the Animal Products (Raw Milk Products Specifications) Notice 2009, an operator must have a registered risk management program which demonstrates that defined food safety criteria are met and includes process measures such as acidification and pH reduction, maturation time and temperature, water activity and salt concentration at different stages of the processing.

The US Food and Drug Administration have compiled a list of outbreaks that occurred from 1987 to September 2010 in the US (FDA, 2011). During this period, there were at least 133 outbreaks that could be attributed to the consumption of raw milk and raw milk products resulting in 2,659 cases of illnesses, 269 hospitalizations, 3 deaths, 6 stillbirths and 2 miscarriages. It is likely that these numbers underestimate the true prevalence, due to under-reporting of illnesses. In 2010, raw milk was associated with at least 8 documented outbreaks in the US; with *Campylobacter*, *Salmonella* and *E. coli* O157:H7 being the identified pathogens (FDA, 2011).

Listeria monocytogenes and *Staphylococcus aureus* are both important food borne pathogens that can be found in raw milk and cheeses, particularly soft cheeses (Little et al, 2008). Milk production forms the first critical control point for the manufacture of raw milk products. Further introduction of pathogens to milk can occur during processing. Processes which influence survival of foodborne pathogens in cheese are, fermentation leading to a significant pH drop, the composition of the starter culture, renneting, salting, and finally ripening (Estrada et al, 1999; Schvartzman et al, 2011; Linton et al, 2008). Lactic acid is an end product of microbial fermentation and can occur in two isoforms, D and L,

depending on microbial species. However, it is only the L-form which has anti-microbial activity (Carr et al. 2002).

Since both *L. monocytogenes* and *S. aureus* are associated with dairy animals and raw milk, they can adversely affect the microbial quality of the milk entering food manufacture without pasteurisation (Little et al, 2008). The aim of this project is to determine the fate of these pathogens during milk fermentation by typical cheese starter cultures. The data collected will enable calibration of new models that describe the effect of chemical changes during fermentation on pathogen dynamics. It should be noted that although the fermentation is performed using cheese starter cultures, this work only examines the fermentation process in liquid milk and does not follow any cheese making protocols that would normally include processes such as renneting, salting or smearing. The project will determine at a high level if the presence of the raw milk microflora affects the survival and growth of pathogens during milk fermentation. It is possible that there is no effect, or that any effect is minimal. If so, then information on pathogen survival and growth in pasteurized milk can be reliably used for assessing risk associated with raw milk products.

1.1 Abbreviations

°C	degrees Celsius
U	units
g	gram
mL	millilitre
L	litre
TSA	tryptic soya agar
TSB	tryptic soya broth
nm	nanometre
cm	centimetre
OD_{600}	optical density at 600nm
T	time point
M	molar
k	thousand
rpm	revolutions per minute
NAD/NADH	Nicotinamide adenine dinucleotide
mg	milligrams
µL	microlitres
Δ	delta
V	final volume
v	sample volume
MW	molecular weight
d	light path
ε	extinction coefficient
mmol	milimoles
cfu	colony forming units
TMTC	too many to count
R	raw milk sample
P	pasteurised milk sample
subsp.	subspecies

2. Materials and Methods

2.1 Bacterial strains and growth conditions

2.1.1 *Listeria monocytogenes* and *Staphylococcus aureus*

Six strains of each pathogenic species were obtained from the Fonterra culture collection to prepare the cocktail inoculum (Table 1). All of these strains have been cultured from milk or milk products and stored under glycerol at -80 °C.

Table 1. Bacterial strains and starter cultures used in this project.

Species	Reference Number	Date of Isolation/Source
L. monocytogenes	605205-4	18-9-2006 / Fonterra
L. monocytogenes	LM227	23-05-2011 / Fonterra
L. monocytogenes	LM232	23-05-2011 / Fonterra
L. monocytogenes	LM04COC9-643	18-09-2006 / Fonterra
L. monocytogenes	LM55787	23-05-2011 / Fonterra
L. monocytogenes	LM425165-9	18-09-2006 / Fonterra
S. aureus	S12	13-09-2010 / Fonterra
S. aureus	S28	07-03-2008 / Fonterra
S. aureus	FM34 (S39)	11-11-2010 / Fonterra
S. aureus	S51	07-03-2008 / Fonterra
S. aureus	S100	12-08-2010 / Fonterra
S. aureus	CB51	09-01-2008 / Fonterra

Cheese Starter Cultures		
Starter Culture A *Lactococcus lactis* subsp. *cremoris;* *L. lactis* subspp *lactis*	FD-DVS pHageControl™ R-704	Chr. Hansen / Fonterra (Chr. Hansen, 2001a)
Starter Culture B *Lactococcus lactis* subspp. *cremoris:* *L. lactis* subspp *lactis:* *L. lactis* subspp *diacetylactis:* *Leuconostoc mesenteroides* subsp. *cremoris*	Flora-Danica	Chr. Hansen / Fonterra (Chr. Hansen, 2001b)

All bacteria cultures except for the starter cultures were grown on Tryptic soya agar (TSA) at 37 °C. Tryptic soya broth (TSB) was used for preparation of broth cultures for cocktail inoculum as required. PALCAM was used as a selective medium at 37 °C to isolate *Listeria* (MIMMS, 2011a). Baird-Parker Agar was used as a selective medium at 37 °C for *Staphylococcus* (MIMMS, 2011b).

2.1.2 Starter cultures

Two commercially available starter cultures used in New Zealand to manufacture cheeses were sourced from Chr. Hansen A/S, in consultation with Fonterra (Table 1).

Cheese starter culture FD_DVS R-704 contains a mixture of *Lactococcus lactis* subspp. *cremoris* and *lactis* and is used in the manufacture of closed texture cheeses such as cheddar and feta (Chr. Hansen, 2001a).

Cheese starter culture FD-DVS Flora-Danica contains a mixture of *L. lactis* subspp. *cremoris, lactis* and *diacetylactis* and *Leuconostoc mesenteroides* subsp. *cremoris* and is used in the manufacture of continental cheeses such as gouda, edam, leerdam as well as some soft cheeses (Chr. Hansen, 2001b).

Starter cultures were prepared and used at the concentration recommended by the manufacturer. Briefly, freeze-dried sachets were stored at -20 °C until required. Sufficient starter culture granules (for 1000L: 50U = 20g is required) were weighed out to achieve the recommended inoculum and resuspended in peptone diluent. The resulting mixture was incubated for 15-20 minutes at room temperature to allow even distribution of the cells prior to inoculating the milk cultures. Bacteria present in the starter culture were isolated using M17 selective growth media (Venter, personal communication).

2.1.3 Pathogen cocktails

Pathogen cocktails, for inoculation into milk, were prepared for both *Listeria* and *Staphylococcus*. Two pathogen inoculum concentrations were required – 10^2 and 10^5 cfu/mL. The low level inoculum only contained one species of the bacteria to ensure equivalence of inoculum between milk samples within and between trials, while the high inoculum contained an equal mix of 6 strains.

Overnight cultures were prepared for each of the six cultures. Optical density at 600 nm (OD_{600}) was measured for each culture. Volume adjustments were made using TSB to normalise culture density. Cocktails were prepared by mixing equal volumes of adjusted overnight cultures together. Actual bacterial numbers in the cocktail per mL were determined using plate counts, and the final cocktail inoculum adjusted to achieve a final cell concentration of approximately 4 x 10^7 cells/mL. An appropriate volume of cocktail was added to each 160 mL aliquot of milk as required. Initial

inoculum levels were determined by plating time zero (T = 0) onto selective media as appropriate.

Low inoculum were prepared in a similar way as the high density cocktail, except that only one strain was used (*S. aureus* - S51; *L. monocytogenes* - LM227). 1 mL of inoculum containing a cell concentration of approximately $4x10^4$ cells/mL was added to each 160 mL aliquot of milk as required. Initial inoculum levels were determined by plating T = 0 onto selective media as appropriate.

2.2 Milk

Matched raw and pasteurised milk samples were obtained locally from the Fonterra Pilot Processing Plant, Grasslands, Palmerston North for this study. Pasteurisation was carried out at the Fonterra Pilot Processing Plant on the same day as milk collection from local farms and compositional data supplied as required. 20 L of each milk were obtained in sterile buckets and stored at +4 °C. 160 mL of milk were dispensed by weight (160 ± 0.1g) into sterile disposable pottles. Aliquoted milk samples were either used immediately or stored overnight at +4 °C. Milk was used within 24 hours post-pasteurisation. In addition, five 20 mL aliquots were taken and frozen at -20 °C for molecular analysis should that be required at a later date.

2.2.1 Antibiotic Testing

Milk was tested prior to use for penicillin-based antibiotics using SNAP* MRL Beta-Lactam Test Kit (IDEXX Laboratories), according to the manufacturers' instructions.

2.3 Lactic acid determinations

During the challenge trials, 3 mL milk samples were collected at hourly intervals for 12 hours from each of the trial ferementations and frozen at -20 °C until analysed.

2.3.1 Pre-treatment of milk samples

Milk samples were pre-treated before lactic acid analysis to remove casein. 0.4 mL 0.2 M sodium acetate (pH 3.95) was added to an equal volume of milk sample and mixed thoroughly. 4.1 mL of sterile distilled H_2O was immediately added to each sample and mixed. Finally, 0.028 mL of 1 M NaOH was added to neutralise the acidic solution to a final pH of approximately 6.0. The resulting mixture was centrifuged at 10k rpm to pellet the casein. 0.2 mL of clear supernatant was transferred to a sterile eppendorf and stored at -20 °C.

2.3.2 Lactic acid determinations

Treated milk samples were analysed for lactic acid using a D-lactic acid/L-lactic acid UV method (Boehringer Mannheim/R-Biopharm Enzymatic Bioanalysis/Food Analysis Kit (Cat. Number: 11 112 821 035)). Lactic acid occurs in two isoforms, D and L. This is determined by the enzymatic pathways used by the bacteria that form the starter culture (Carr et al. 2002). Both forms of lactic acid are detectable using this determination method.

96-well based assays were undertaken according to the manufacturer's instructions with modification. All solutions and suspensions are provided in the kit. To each well, 100 µL of glycylglycine buffer pH 10.0 with 440 mg glutamic acid (Solution 1), 20 µL 35 mg/mL NAD (Solution 2), 2 µL glutamate-pyruvate transaminase (1100 U) (Suspension 3), 100 uL redistilled water, and 10 uL pre-treated sample (Section 3.3.1) were added and mixed. Following a 5 minute incubation at 25 °C, absorbance values were read at 340 nm (Reading A1). The enzymatic reaction was started by the addition of 2 µL of D-lactate dehydrogenase (3800 U) (Solution 4). After a further incubation of 30 minutes at 25 °C, the absorbance readings of blanks and samples were taken (Reading A2). To complete the assay 2 µL of L-lactate dehydrogenase (3800 U) (Solution 5) was added and a final incubation of 30 minutes at 25 °C was completed prior to the final absorbance reading (A3).

Calculations to determine absorbance and ultimately the lactic acid concentration were carried out as follows.

The absorbance differences (A2-A1) were determined for both blank and sample thereby obtaining $\Delta A_{\text{D-lactic acid}}$

The absorbance differences (A3-A2) were determined for both blank and sample thereby obtaining $\Delta A_{\text{L-lactic acid}}$

According to the general equation for calculating concentration:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A \text{ (g/L)}$$

Where:

V	=	final volume (mL)
v	=	sample volume (mL)
MW	=	molecular weight of the substance to be assayed (g/L)
d	=	light path (cm)
ε	=	extinction coefficient of NADH at 340 nm = 6.3 (L x $mmol^{-1}$ x cm^{-1})

Therefore for D-lactic acid calculations:

$$c = \frac{0.224 \times 90.1}{\varepsilon \times 1 \times 0.01 \times 1000} \times \Delta A \text{ (g/L)}$$

$$= \frac{0.2018}{6.3} \times \Delta A \text{ [g D-lactic acid/L sample solution]}$$

Therefore for L-lactic acid calculations:

$$c = \frac{0.226 \times 90.1}{\varepsilon \times 1 \times 0.01 \times 1000} \times \Delta A \text{ (g/L)}$$

$$= \frac{0.2036}{6.3} \times \Delta A \text{ [g L-lactic acid/L sample solution]}$$

Finally, results were multiplied by the sample dilution factor of 12.5.

2.4 Lactose Assays

Pre-treated milk samples (see Section 2.3.1) were analysed for lactose using a Lactose/D-galactose UV method (Boehringer Mannheim/R-Biopharm Enzymatic Bioanalysis/Food Analysis Kit (Cat. Number: 10 176 303 035)). Assays were undertaken according to the manufacturer's instructions, using solutions and suspensions provided in the kit.

2.5 Statistical Analysis

The statistical analysis performed explored whether there were significant differences between raw and pasteurised milk for a experimental parameters over the various hours of incubation. Since repeated measurements have been considered for each of the responses of interest (e.g. pH, lactic acid and bacterial count), a 'Repeated Measures ANOVA' approach was implemented together with suitable graphical representation of the data (Appendix 2). Two experimental factors, 'type of milk' and the repeated measures factor 'time', have been employed in the ANOVA procedure. This analysis was carried out separately for each of the 16 different scenarios of pathogen (*S. aureus* or *L. monocytogenes*), inoculum level (high or low), starter culture (A or B), and challenge trial (1 or 2). Furthermore, existence of significant differences between raw and pasteurised milk at the start of the incubation (T = 0 hours) and after 12 hours (T = 12) of incubation were examined.

Caveat: The conclusions based on the available data should be treated with caution as there are only two independent replicates for each of the raw and pasteurised milk in each of the 16 different scenarios considered.

Consequently, the conclusions made should be regarded as ‘descriptive’ or ‘preliminary observations’.

There were missing values associated with bacterial count for some replicates at some time points. With only two replicates, some with missing values, for simplicity and completion of the ANOVAs, the missing values were substituted with the values of the corresponding non-missing replicate.

3. Protocol Design

This report focuses on the results generated from the two challenge trials that followed on from the initial pilot study. A summary of the statistical analysis carried out on data from both challenge trials is listed in the Appendix.

The overall experimental design and sampling plan for these challenge trials are outlined in Figures 1 and 2. Ross (2011) and data gathered during the pilot trial were used as a basis for developing and modifying the protocol used (Withers and Couper, 2011).

Two independent challenge trials were performed in this study. Matched raw and pasteurised whole milk samples (minimum 2 lots of 10 L each to limit temperature variations) were sourced from the Fonterra Pilot Plant at Grasslands, Palmerston North. Raw milk was collected each morning from local farms and transported by tanker to the processing plant, where it was processed the same day. Bulk milk for the trial was collected in the morning and stored at +4 °C in chillers at the Hopkirk Research Institute, until required. Experimentation was performed within 24 hours of pasteurisation. Both milk batches were tested for the presence of antibiotics and for the presence of test pathogens, *S. aureus* and *L. monocytogenes.* Milk was aliquoted into 160 mL aliquots, in large pottles and stored at +4 °C chillers until required. Specific milk details for each part of the challenge trials are detailed in the relevant sections.

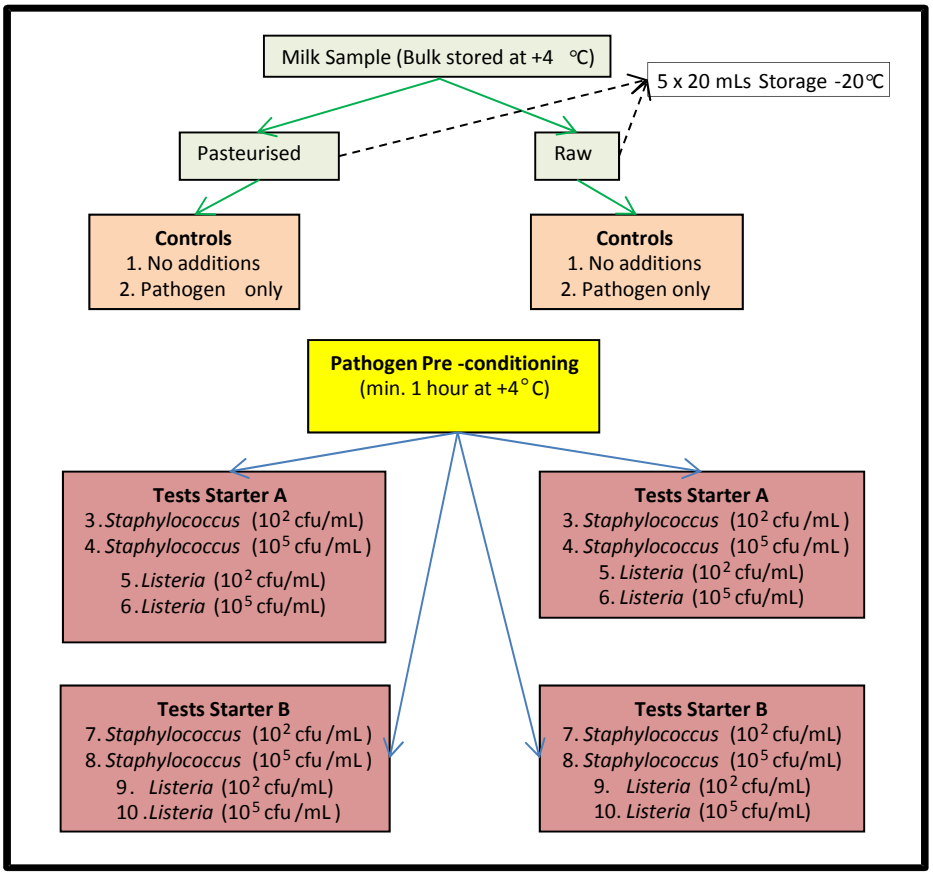

Figure 1. Overall summary schematic diagram of the project experimental design.

To mimic the effect of milk storage at +4 °C on pathogenic bacteria, a conditioning period was carried out. Pathogenic bacteria were added to appropriate milk aliquots one hour prior to the addition of starter cultures to allow pathogen conditioning (Figure 1). 10^2 cfu/mL inoculum comprised a single representative strain while the 10^5 cfu/mL inoculum was a cocktail of six strains milk-associated isolates. Once pathogen pre-conditioning was completed, starter cultures were added to the milk, as indicated, at the concentration and in the manner recommended by the manufacturer. All milk cultures were incubated at 30 °C with gentle agitation of 80 rpm. Samples were taken hourly from each milk culture for microbiological, pH and chemistry analyses. Milk cultures were well mixed prior to samples being withdrawn. Microbial analysis and pH were completed immediately after samples were taken. Samples for chemistry were stored at -20 °C until processed.

Two milk aliquots were used for each condition in the trial and data presented is the average of these two independent samples.

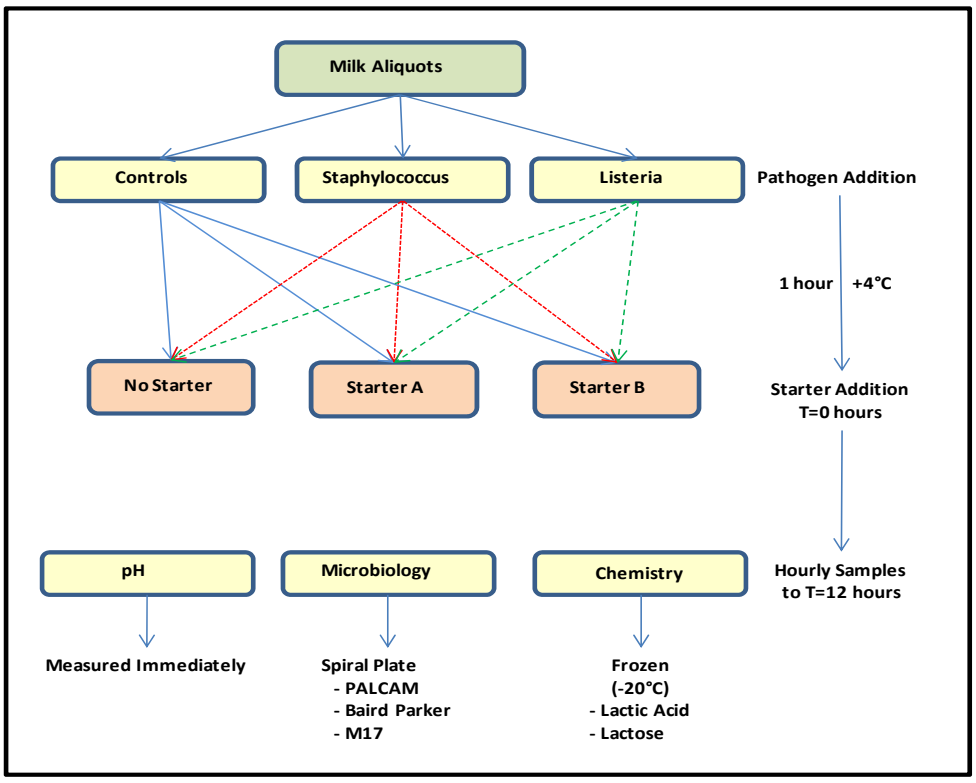

Figure 2. Schematic diagram of sample handling in the trials.

4. Results

4.1 *Staphylococcus aureus* Challenge Trials 1 and 2

S. aureus challenge trial one was carried out using the same raw and pasteurised milks over two consecutive days (Day 1 and Day 2), within 24-hours of pasteurisation,. Milk composition was the same for both raw and pasteurised milk (Table 2).

Challenge trial two was carried out in two stages (High Inoculum (HI) and Low Inoculum (LI)) using two independent raw and pasteurised milk samples (Table 2). Initial β-lactam antibiotic testing of all milk samples showed no detectable antibiotics that would inhibit either starter culture or pathogen growth.

Table 2. Milk composition for *S. aureus* challenge trials 1 and 2

Component	*Percentage (%)		
	Challenge trial 1	Challenge trial 2 - HI	Challenge trial 2 - LI
Fat	5.09	5.25	5.38
Protein	3.54	3.68	3.78
Lactose	4.67	4.68	4.78
Total Solids	13.87	14.22	14.54

*Data supplied by Fonterra

4.1.1 *S. aureus* Challenge Trial 1

Lactic acid bacteria (LABs) were detected in the raw milk used in trial 1 ($6.7 \times 10^3 \pm 7.1 \times 10^2$ cfu/mL and $8.9 \times 10^3 \pm 5.6 \times 10^3$ cfu/mL, Day 1 and Day 2 respectively).

No *S. aureus* was recovered from the pasteurised milk, however *S. aureus* was present in the raw milk sample ($1.1 \times 10^3 \pm 7.4 \times 10^2$ cfu/mL for Challenge Trial 1 on Day 1 and $8.9 \times 10^3 \pm 5.6 \times 10^3$ cfu/mL on Day 2 (Figure 3B lower graph blue line). A 2.5 log increase in naturally occurring *S. aureus* numbers was observed over the 12-hour incubation in raw milk (Figure 3B – lower graph blue line).

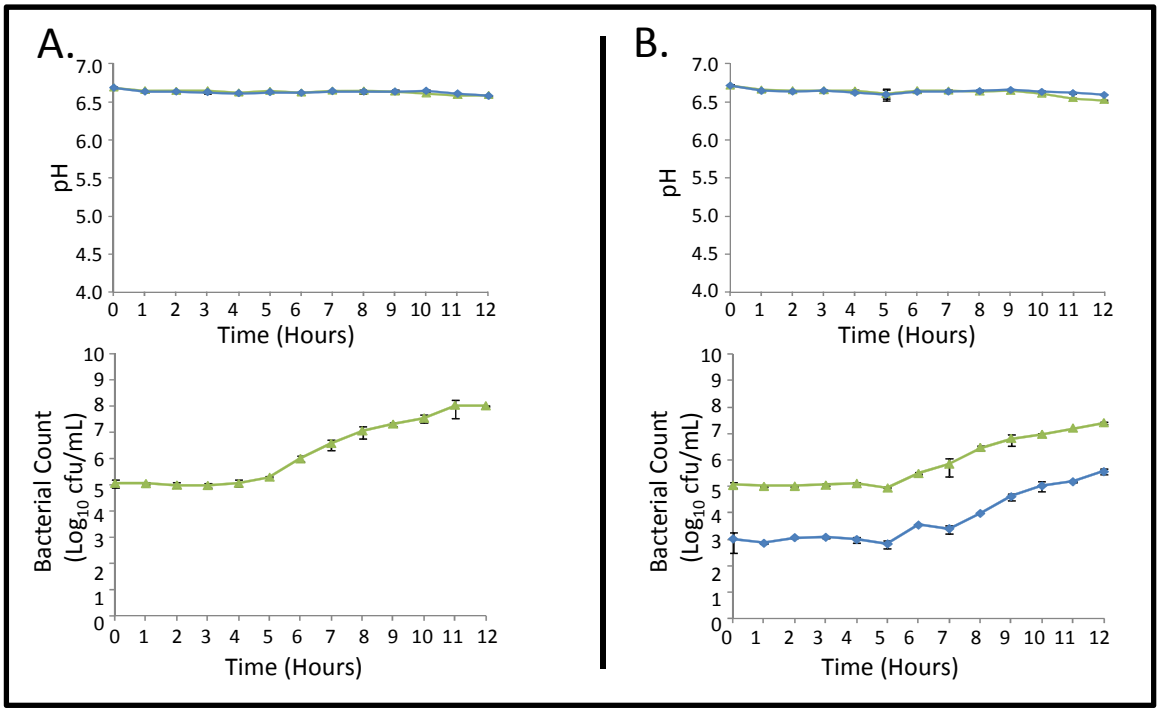

Figure 3. *S. aureus* growth in pasteurised (A) and raw (B) milk at 30 °C.

No addition control - Blue diamonds.
S. aureus (approximately 10^5 cfu/mL) added – Green triangles.
Data is presented as the average of duplicate milk aliquots. Error bars are ±1 standard deviation.

Initial pH of both raw and pasteurised milk was 6.71 ± 0.01 and 6.68 ± 0.01 respectively. No significant change in pH was observed over the 12-hours of incubation for both milk types with no addition or when only *S. aureus* was added (Figure 3 A and B – upper graphs). After 12 hours of incubation at 30 °C, the pH of raw and pasteurised milk was 6.58 and in the presence of *S. aureus,* 6.52 and 6.58 respectively. No lactic acid was detected in any of these milk aliquots over the 12-hour period (data not shown).

S. aureus was added to both raw and pasteurised milks with a starting inoculum of approximately 10^5 cells/mL to determine pathogen growth in milk. In both milk types, *S. aureus* numbers increased over the 12-hours of the experiment (3 log and 2.5 log in pasteurised and raw milks respectively). In both cases, a 4- to 5- hour lag period occurred before cell numbers were observed to increase (Figure 3 – lower graphs).

Two different commercial cheese starter cultures were used in this trial. Freeze-dried starter cultures were prepared in peptone water and allowed to fully rehydrate before being added to milk samples as appropriate. Fermentation incubation temperature was 30 °C as this was at higher end of the optimal range for both starter cultures growth.

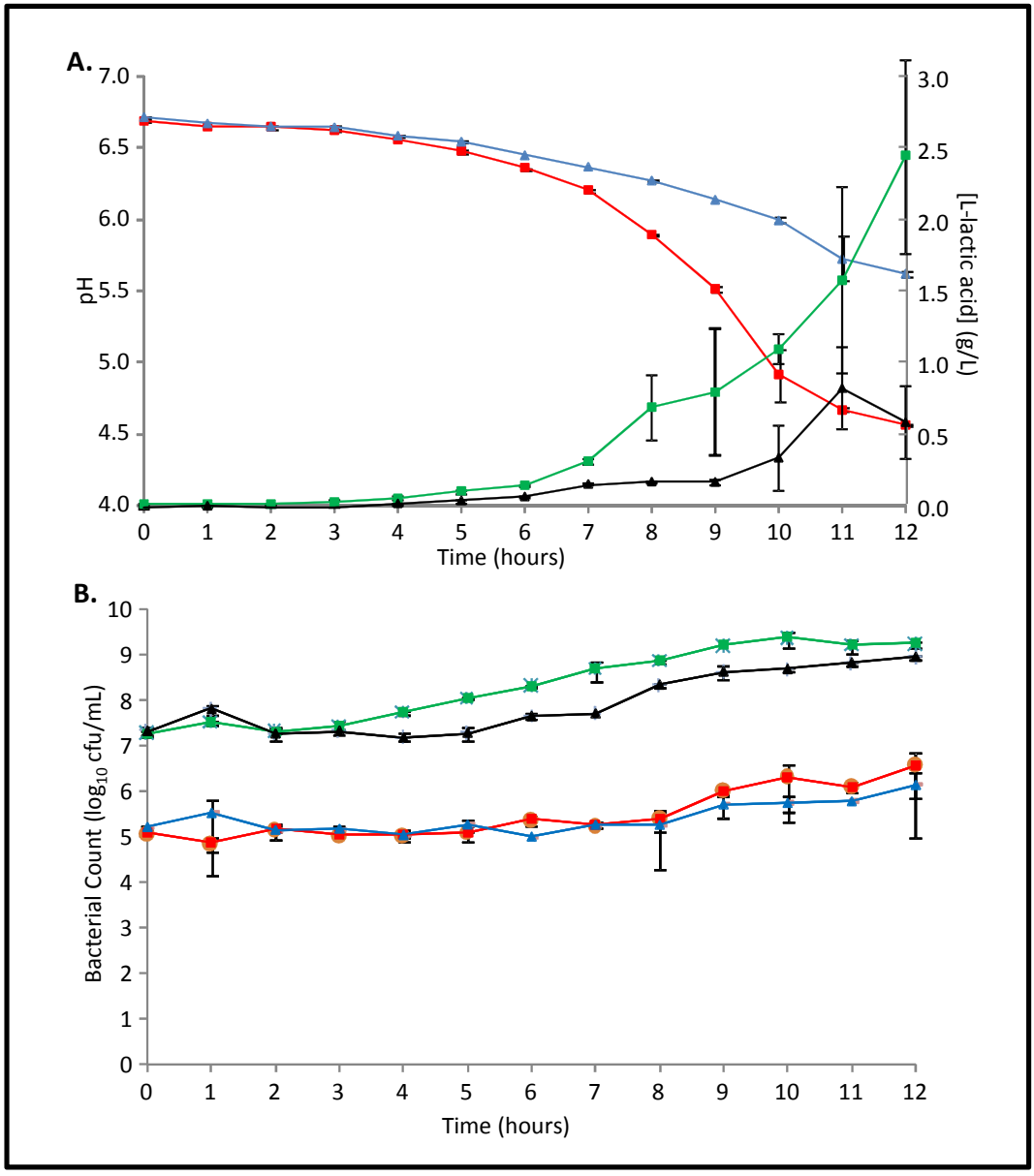

Figure 4. Effect of cheese starter culture A on growth and survival of a high *S. aureus* inoculum. Assays were performed in matched pasteurised and raw milks.

A. pH and L-lactic acid concentration

- Raw Milk – pH
- Pasteurised Milk - pH
- Raw Milk – L-lactic acid concentration
- Pasteurised Milk – L-lactic acid concentration

B. Bacterial growth during 12-hour milk fermentation

- Raw Milk – Starter culture
- Pasteurised Milk – Starter culture
- Raw Milk – *S. aureus*
- Pasteurised Milk – *S. aureus*

Data is presented as the average of duplicate milk aliquots. Error bars are ±1 standard deviation.

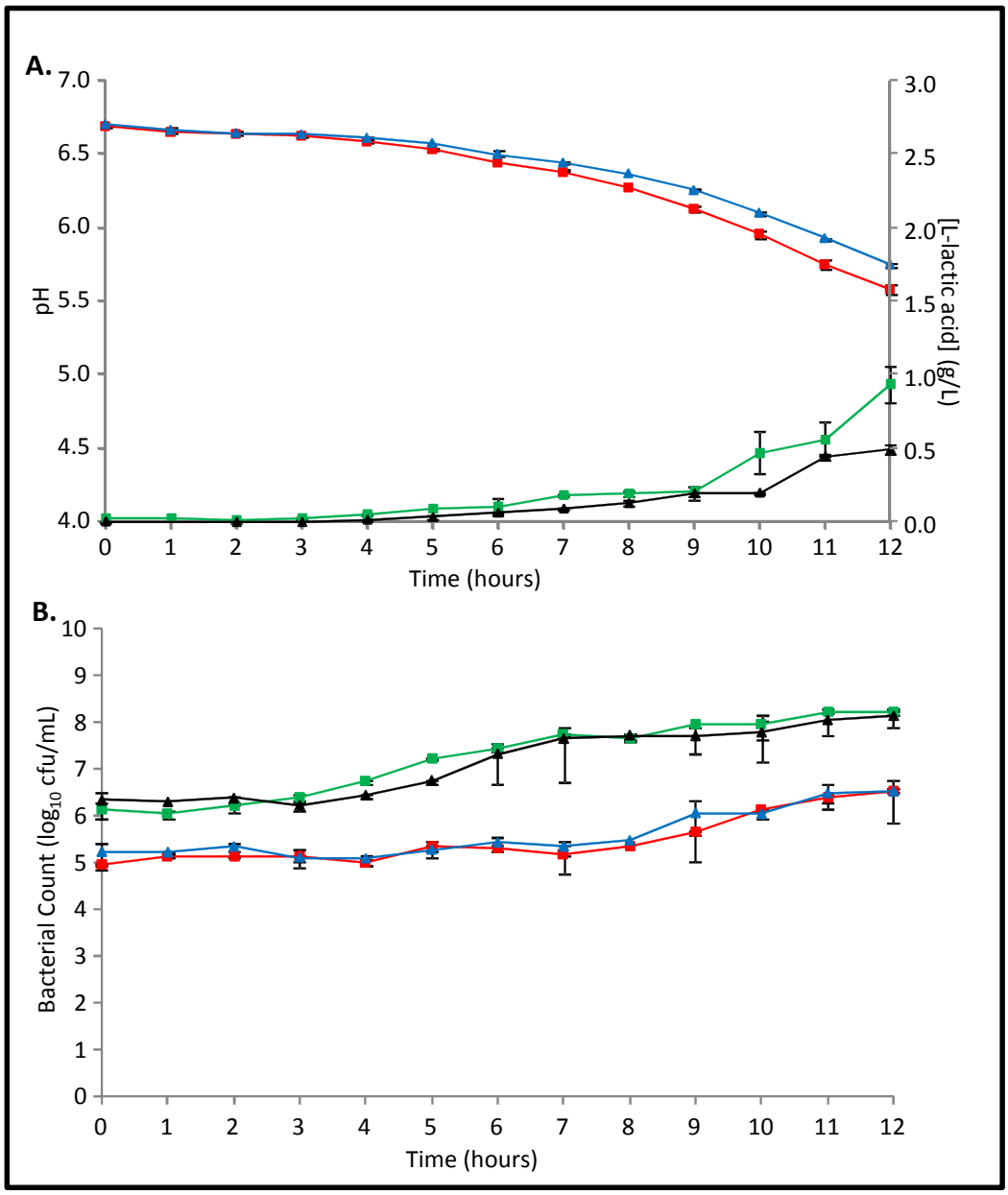

Figure 5. Effect of cheese starter culture B on growth and survival of a high *S. aureus* inoculum. Assays were performed in matched pasteurised and raw milks.

A. pH and L-lactic acid concentration

- Raw Milk – pH
- Pasteurised Milk - pH
- Raw Milk – L-lactic acid concentration
- Pasteurised Milk – L-lactic acid concentration

B. Bacterial growth during 12-hour milk fermentation

- Raw Milk – Starter culture
- Pasteurised Milk – Starter culture
- Raw Milk – *S. aureus*
- Pasteurised Milk – *S. aureus*

Data is presented as the average of duplicate milk aliquots. Error bars are ±1 standard deviation.

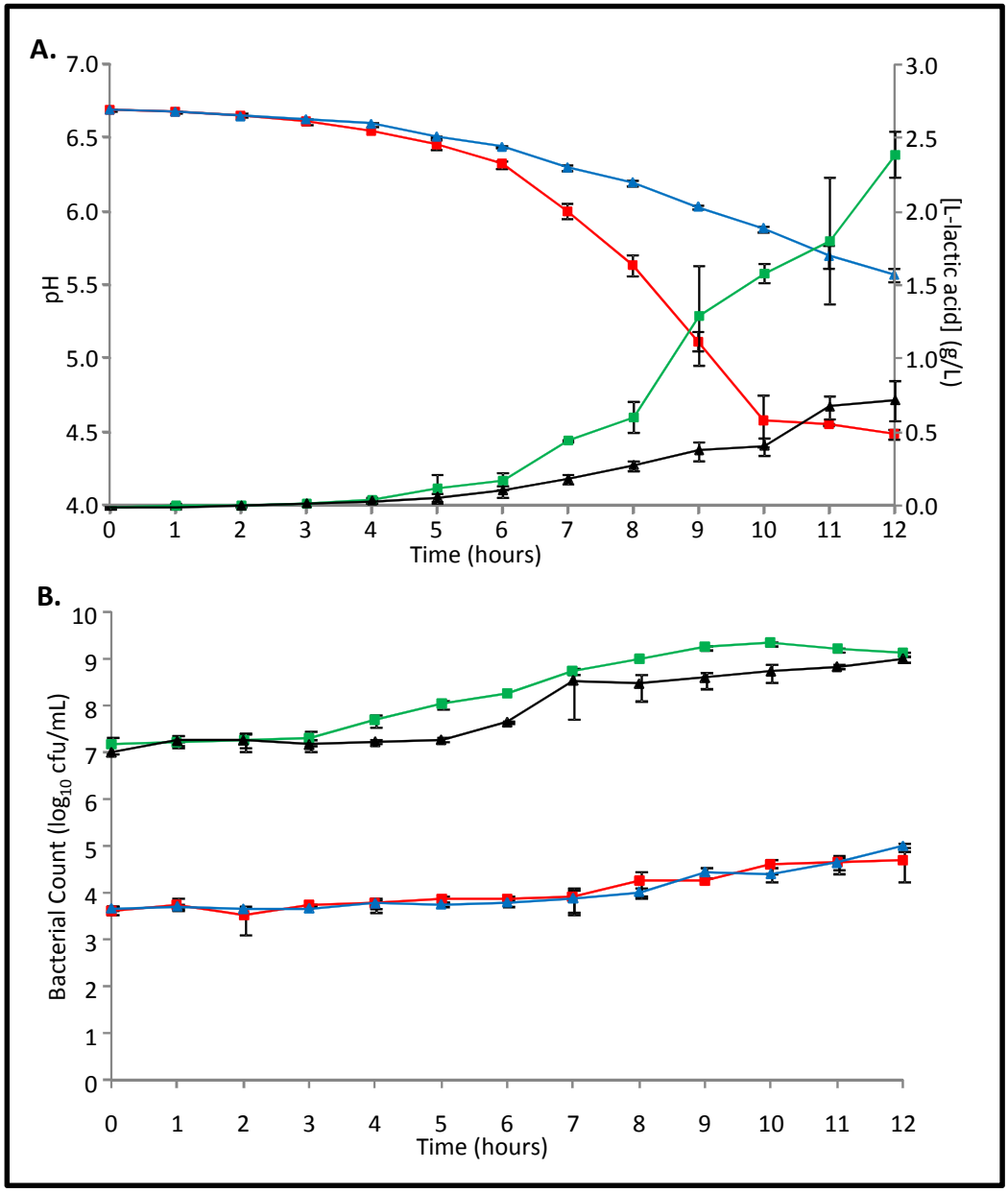

Figure 6. Effect of cheese starter culture A on growth and survival of a low *S. aureus* inoculum. Assays were performed in matched pasteurised and raw milks.

A. pH and L-lactic acid concentration

- Raw Milk – pH
- Pasteurised Milk - pH
- Raw Milk – L-lactic acid concentration
- Pasteurised Milk – L-lactic acid concentration

B. Bacterial growth during 12-hour milk fermentation

- Raw Milk – Starter culture
- Pasteurised Milk – Starter culture
- Raw Milk – *S. aureus*
- Pasteurised Milk – *S. aureus*

Data is presented as the average of duplicate milk aliquots. Error bars are ±1 standard deviation

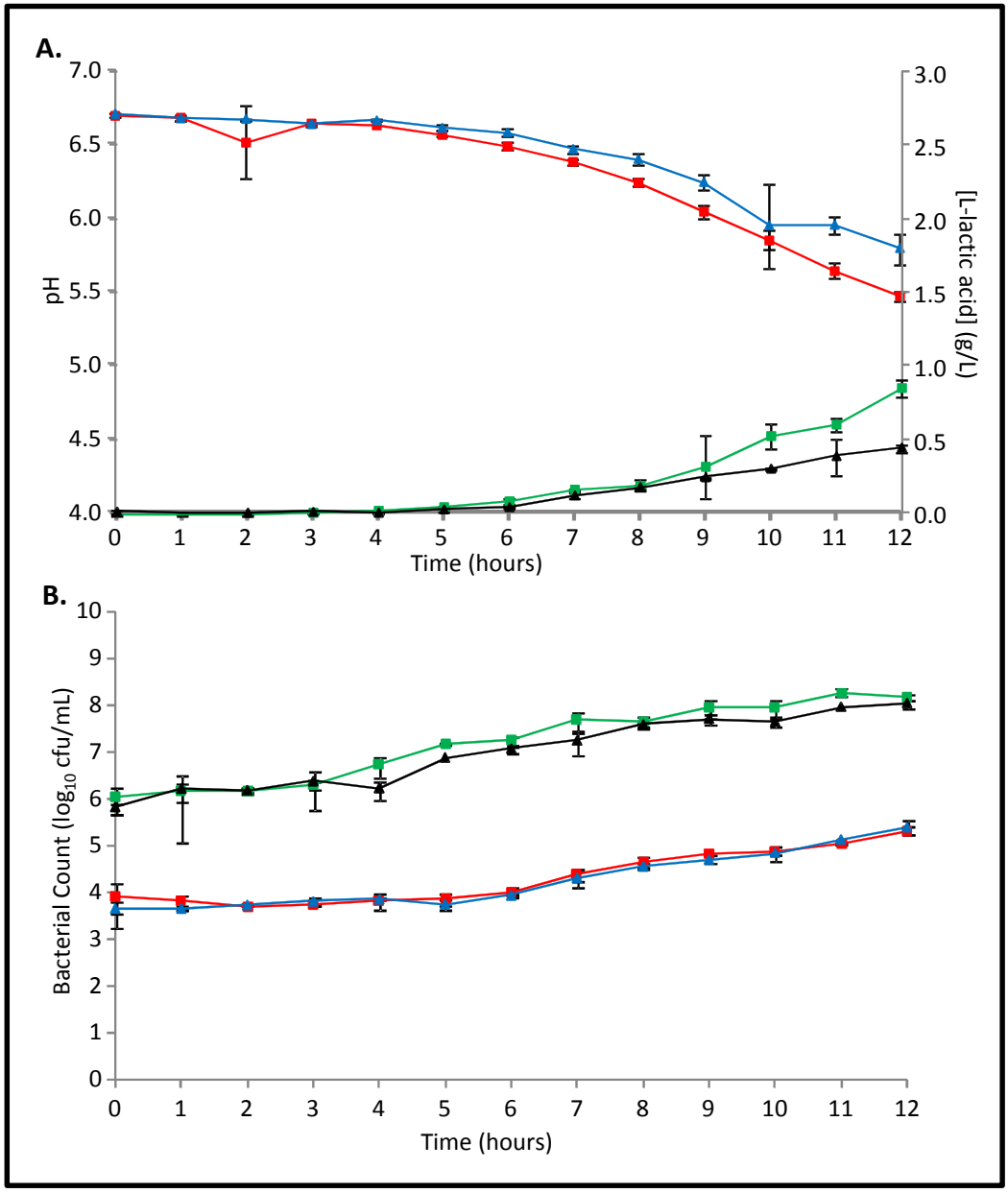

Figure 7. Effect of cheese starter culture B on growth and survival of a low *S. aureus* inoculum. Assays were performed in matched pasteurised and raw milks.

A. pH and L-lactic acid concentration

- Raw Milk – pH
- Pasteurised Milk - pH
- Raw Milk – L-lactic acid concentration
- Pasteurised Milk – L-lactic acid concentration

B. Bacterial growth during 12-hour milk fermentation

- Raw Milk – Starter culture
- Pasteurised Milk – Starter culture
- Raw Milk – *S. aureus*
- Pasteurised Milk – *S. aureus*

Data is presented as the average of duplicate milk aliquots. Error bars are ±1 standard deviation.

Two inoculum concentrations were used in this trial – a high inoculum of 10^5 and a low inoculum of 10^2 cfu/mL. *S. aureus* was added to appropriate milk aliquots 1-hour prior to the addition of starter cultures to allow the bacteria to adapt to the milk. This conditioning period was carried out at +4 °C to mimic milk storage. The final inoculum added were determined at the T = 0 time point. For the high inoculum, the final *S. aureus* inoculum for this trial was 1.2 x 10^5, 8.5 x 10^4 and 1.2 x 10^5 cfu/mL in pasteurised milk (pathogen only, Starter A and Starter B samples respectively) and 1.1 x 10^5, 1.6 x 10^5 and 1.5 x 10^5 cfu/mL raw milk (pathogen only, Starter A and Starter B sample respectively). For the low inoculum, the final *S. aureus* inoculum was 8 x 10^3, 4.2 x10^3 (Starter A and Starter B samples respectively) in pasteurised milk and 4.5 x 10^3 and 4.6 x10^3 cfu/mL in raw milk (Starter A and B samples respectively).

In the presence of both starter cultures, pH was observed to decrease after approximately 5 hours of incubation. This pH decrease coincided with an increase in concentration of L-lactic acid (Figures 4A, 5A, 6A and 7A). No D-lactic acid was detected in any of the milk samples (data not shown). Starter culture A produced more L-lactic acid in the pasteurised milk than in raw milk (Figures 4A and 6A), resulting in lower pH readings by T = 12 hours. Starter B produced similar concentrations of L-lactic acid, resulting in similar final pH readings (Figure 5A and 7A).

Both starter culture numbers increased by approximately two logs over the 12-hour fermentation in both raw and pasteurised milks alike (Figures 4A, 5A, 6A and 7A). Growth rates during the exponential period for each starter culture were similar regardless of type of milk. This suggested that the naturally occurring microflora in raw milk did not influence starter culture growth per se.

For the high *S. aureus* inoculum, over the 12-hour fermentation, a similar increase of approximately 1.5 logs in *S. aureus* cell numbers was observed, regardless of type of milk or starter culture used (Figures 4B and 5B). In both cases, the lag period observed increased from the 5 hours without any starter culture to 8 hours with starter culture (Figures 3, 4B and 5B). Although, *S. aureus* lag time was longer in the presence of starter cultures, similar growth rates were observed during exponential phase of growth, suggesting the observed difference in final bacterial numbers observed at T = 12 was as a consequence of the longer lag rather than a change in growth capability.

Similar increases in cell numbers were observed for the low *S. aureus* inoculum, with increases in cell number of approximately 1 to 1.5 logs, regardless of type of milk or starter culture used (Figures 6B and 7B).Furthermore, no differences in growth were observed as a response to increased levels of L-lactic acid and the associated decrease in pH.

4.1.2. *S. aureus* Challenge Trial 2

S. aureus challenge trial 2 was performed using two independent milk samples, HI and LI (Table 2). Lactic acid bacteria (LABs) were detected in the raw milk ($6.6 \times 10^3 \pm 5.5 \times 10^2$ and $2.2 \times 10^3 \pm 1.5 \times 10^2$ cfu/mL HI and LI respectively). Lactic acid bacteria were detected in the pasteurised milk at the start of this trial as had been previously observed but again below quantification levels.

No *S. aureus* was initially recovered from either batch of pasteurised milk, however *S. aureus* was present in both raw milk samples ($1.3 \times 10^3 \pm 5.9 \times 10^2$ cfu/mL (HI) and $4.2 \times 10^2 \pm 6.1 \times 10^1$ cfu/mL (LI); Figure 8B). A 2.5 log increase in *S. aureus* numbers was observed in raw milk (Figures 8B and 8D – lower graph). This was in agreement with observations made in Challenge trial 1.

Initial pH of both raw and pasteurised milk for HI trial was 6.67 ± 0.02 and for the LI trial 6.66 ± 0.02 and 6.64 for raw and pasteurised respectively. No significant change in pH was observed over the 12 hours of incubation for both milk types with no additions or when only *S. aureus* was added (Figure 8 A-D – upper graphs). After 12 hours of incubation at 30 °C, the pH of raw and pasteurised milk was 6.57 and 6.58 respectively for HI and 6.55 and 6.59 respectively for LI and in the presence of *S. aureus,* 6.54 and 6.50 raw and pasteurised respectively. No lactic acid was detected in any of these milk aliquots over the 12-hour incubation period (data not shown).

S. aureus was added to both raw and pasteurised milk with a starting inoculum of 6.6×10^4 and 1.1×10^5 cells/mL respectively, to determine pathogen growth in milk without the addition of starter cultures (Figures 8A and B). In both milk types, *S. aureus* numbers increased over the 12 hours of the experiment (3 log and 2.5 log in pasteurised and raw milks respectively). In both cases, a 4- or 5- hour lag period occurred before cell numbers were observed to increase in pasteurised and raw milk respectively (Figure 8 A and B – lower graphs). This lag period was also observed for the naturally occurring *S. aureus* (Figure 8 B and D - lower graphs).

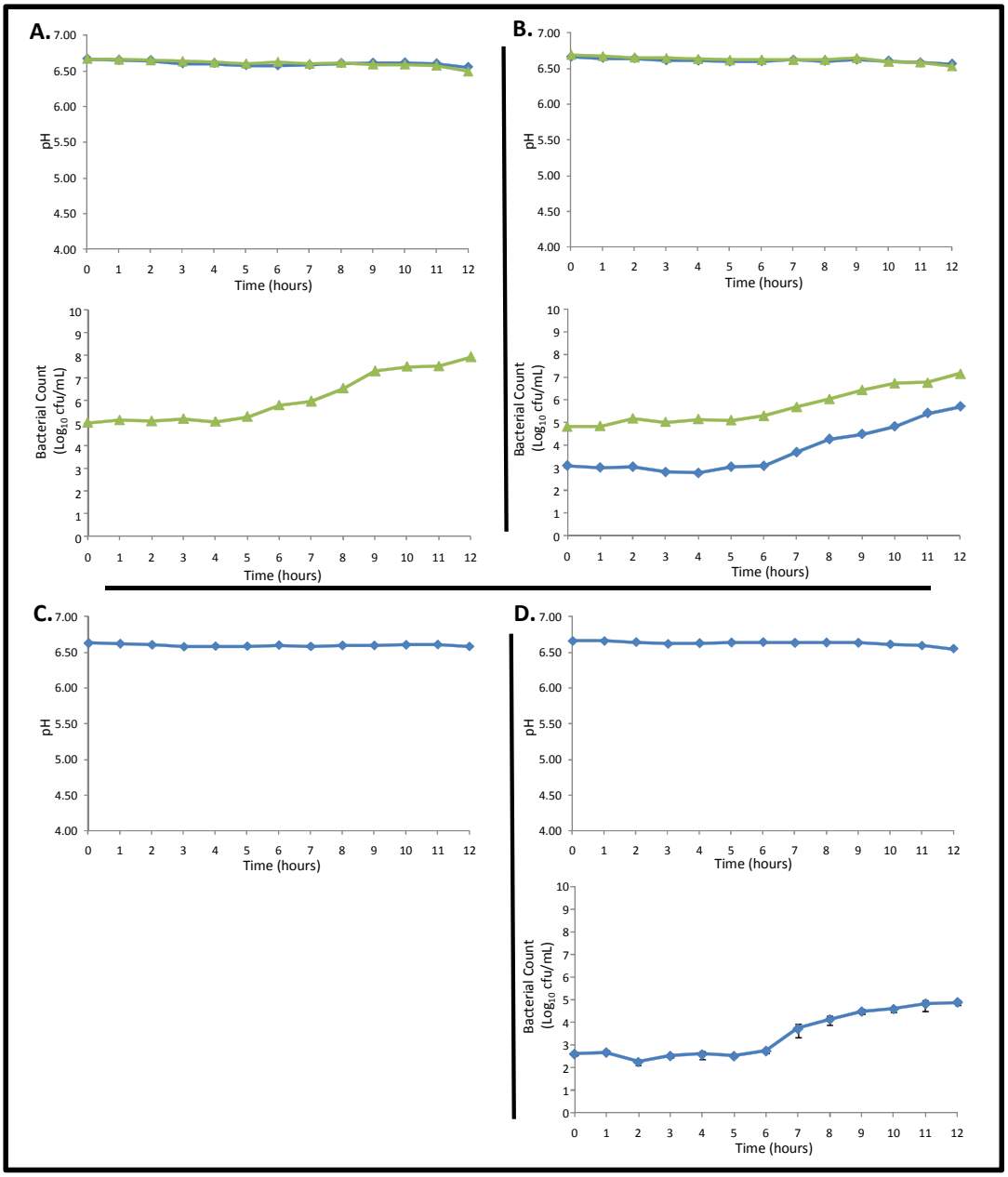

Figure 8. *S. aureus* growth in pasteurised (A, C) and raw (B, D) milk at 30 °C. A and B were from milk sample HI, while C, D were from milk sample LI.

No addition control- Blue diamonds.
S. aureus (approximately 10^5 cfu/mL) added – Green triangles (A and B only).
Data is presented as the average of duplicate milk aliquots. Error bars are ±1 standard deviation.

As for challenge trial 1, freeze-dried starter cultures were prepared in peptone water and allowed to fully rehydrate before being added to milk samples as appropriate. Fermentation incubation temperature was held at 30 °C.

In challenge trial two, the average final high inoculum were $6.7 \times 10^4 \pm 5.4 \times 10^3$ and $1.2 \times 10^5 \pm 1.3 \times 10^4$ cfu/mL in pasteurised and raw milk respectively (Figure 7). For the low inoculum, the average final inoculum was $2.5 \times 10^2 \pm 1.5 \times 10^2$ and $4.0 \times 10^2 \pm 7.8 \times 10^1$ cfu/mL in pasteurised and raw milk respectively (Figures 9 -12).

In the presence of both starter cultures, pH was observed to decrease after 6 hours of incubation. This pH decrease coincided with an increase in concentration of lactic acid (Figure 9A, 10A, 11A and 12A). Starter culture A produced more lactic acid than Starter culture B, which resulted in a lower final pH in milk samples containing Starter culture A. However, in raw milk, both starter cultures produced less lactic acid, which resulted in a smaller pH decrease.

In both HI and LI, starter culture numbers increased by approximately 2 logs over the fermentation period (Figures 9 -12 B). Growth rates during the exponential phase for each starter were similar regardless of the type of milk used.

For the high *S. aureus* inoculum, a similar increase in *S. aureus* cell numbers to that observed in challenge trial 1 of approximately 1-2 logs was observed over the 12-hour fermentation, regardless of type of milk or starter culture used (Figure 9B and 10B).

Similar increases in cell numbers were observed for the low *S. aureus* inoculum, with increases in cell number of approximately 1 to 2 logs, regardless of type of milk or starter culture used (Figure 11B and 12B).

In all cases, regardless of inoculum size, a 5 to 6 hour lag period was observed before cell numbers increased and an exponential growth period occurred.

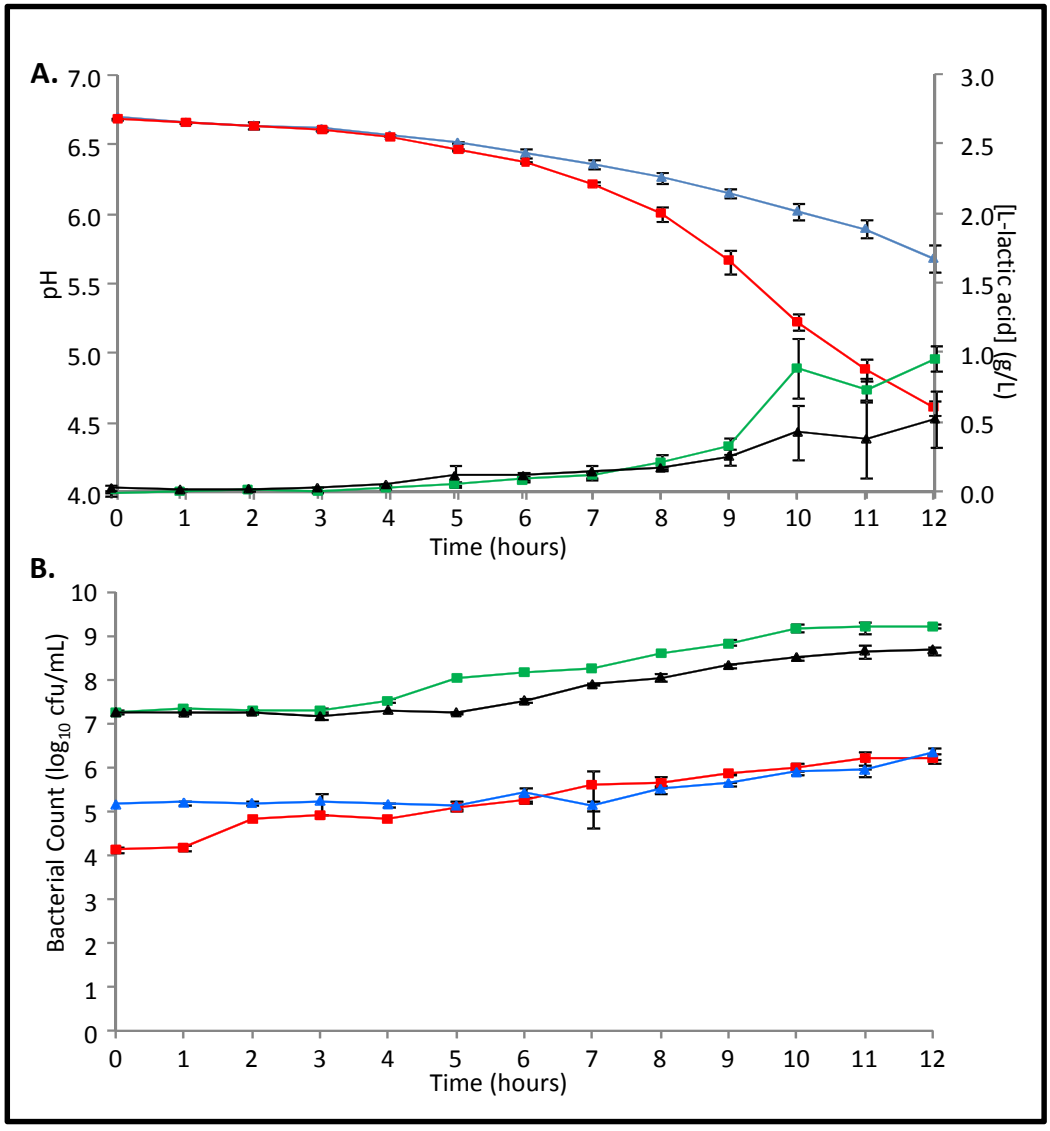

Figure 9. Effect of cheese starter culture A on growth and survival of a high *S. aureus* inoculum. Assays were performed in matched pasteurised and raw milks.

A. pH and L-lactic acid concentration

- Raw Milk – pH
- Pasteurised Milk - pH
- Raw Milk – L-lactic acid concentration
- Pasteurised Milk – L-lactic acid concentration

B. Bacterial growth during 12-hour milk fermentation

- Raw Milk – Starter culture
- Pasteurised Milk – Starter culture
- Raw Milk – *S. aureus*
- Pasteurised Milk – *S. aureus*

Data is presented as the average of duplicate milk aliquots. Error bars are ±1 standard deviation.

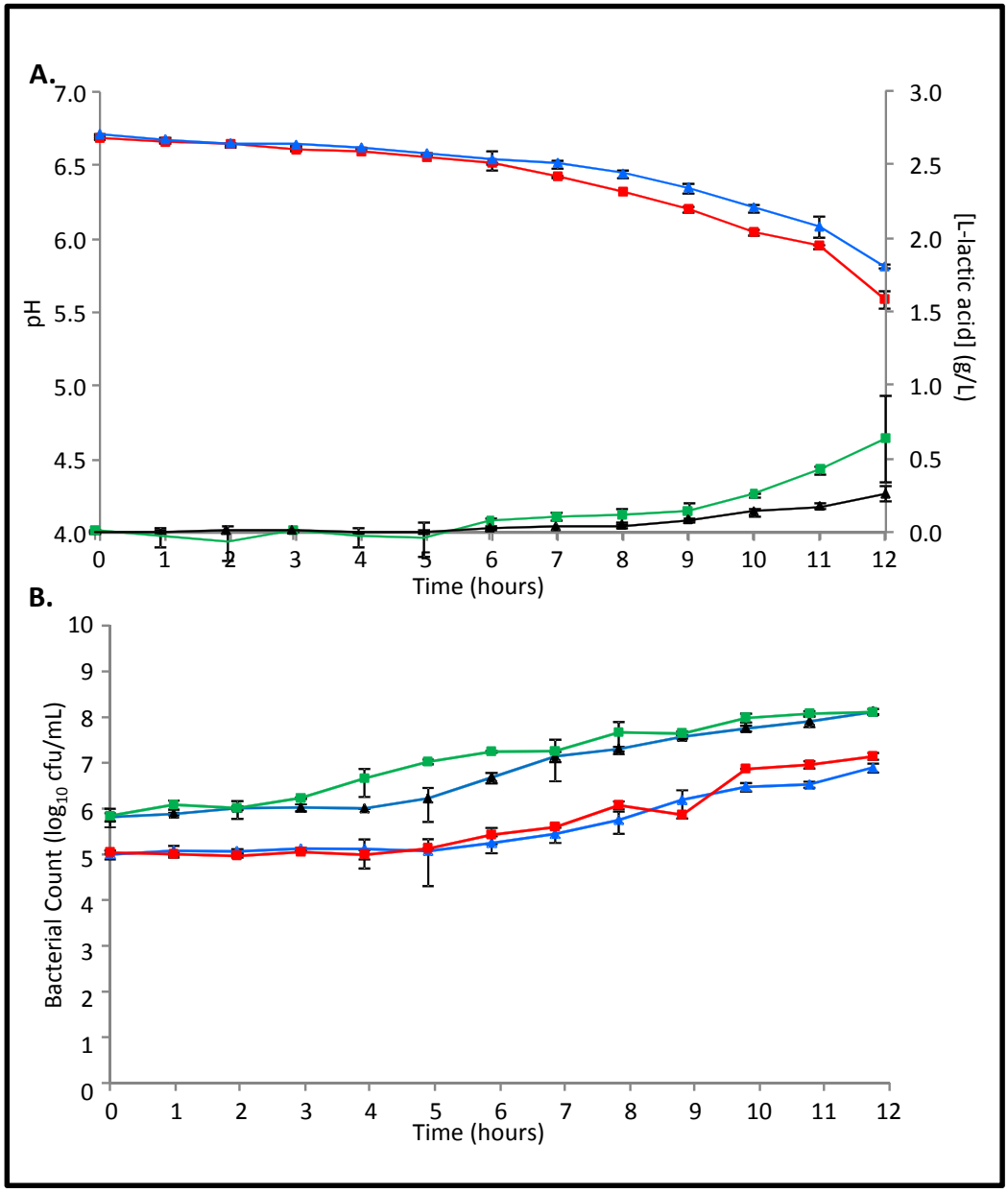

Figure 10. Effect of cheese starter culture B on growth and survival of a high *S. aureus* inoculum. Assays were performed in matched pasteurised and raw milks.

A. pH and L-lactic acid concentration

- Raw Milk – pH
- Pasteurised Milk - pH
- Raw Milk – L-lactic acid concentration
- Pasteurised Milk – L-lactic acid concentration

B. Bacterial growth during 12-hour milk fermentation

- Raw Milk – Starter culture
- Pasteurised Milk – Starter culture
- Raw Milk – *S. aureus*
- Pasteurised Milk – *S. aureus*

Data is presented as the average of duplicate milk aliquots. Error bars are ±1 standard deviation.

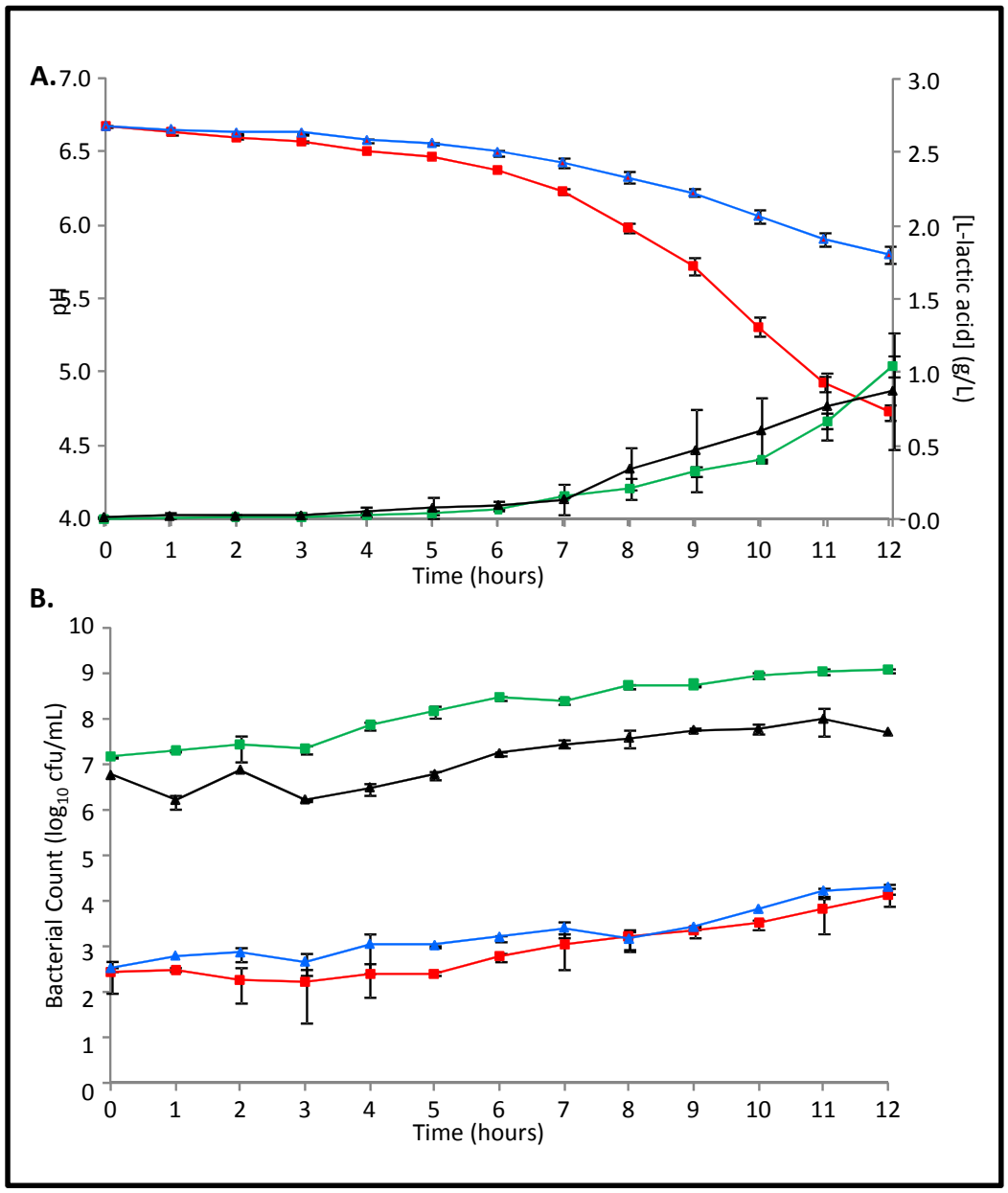

Figure 11. Effect of cheese starter culture A on growth and survival of a low *S. aureus* inoculum. Assays were performed in matched pasteurised and raw milks.

A. pH and L-lactic acid concentration

- Raw Milk – pH
- Pasteurised Milk - pH
- Raw Milk – L-lactic acid concentration
- Pasteurised Milk – L-lactic acid concentration

B. Bacterial growth during 12-hour milk fermentation

- Raw Milk – Starter culture
- Pasteurised Milk – Starter culture
- Raw Milk – *S. aureus*
- Pasteurised Milk – *S. aureus*

Data is presented as the average of duplicate milk aliquots. Error bars are ±1 standard deviation

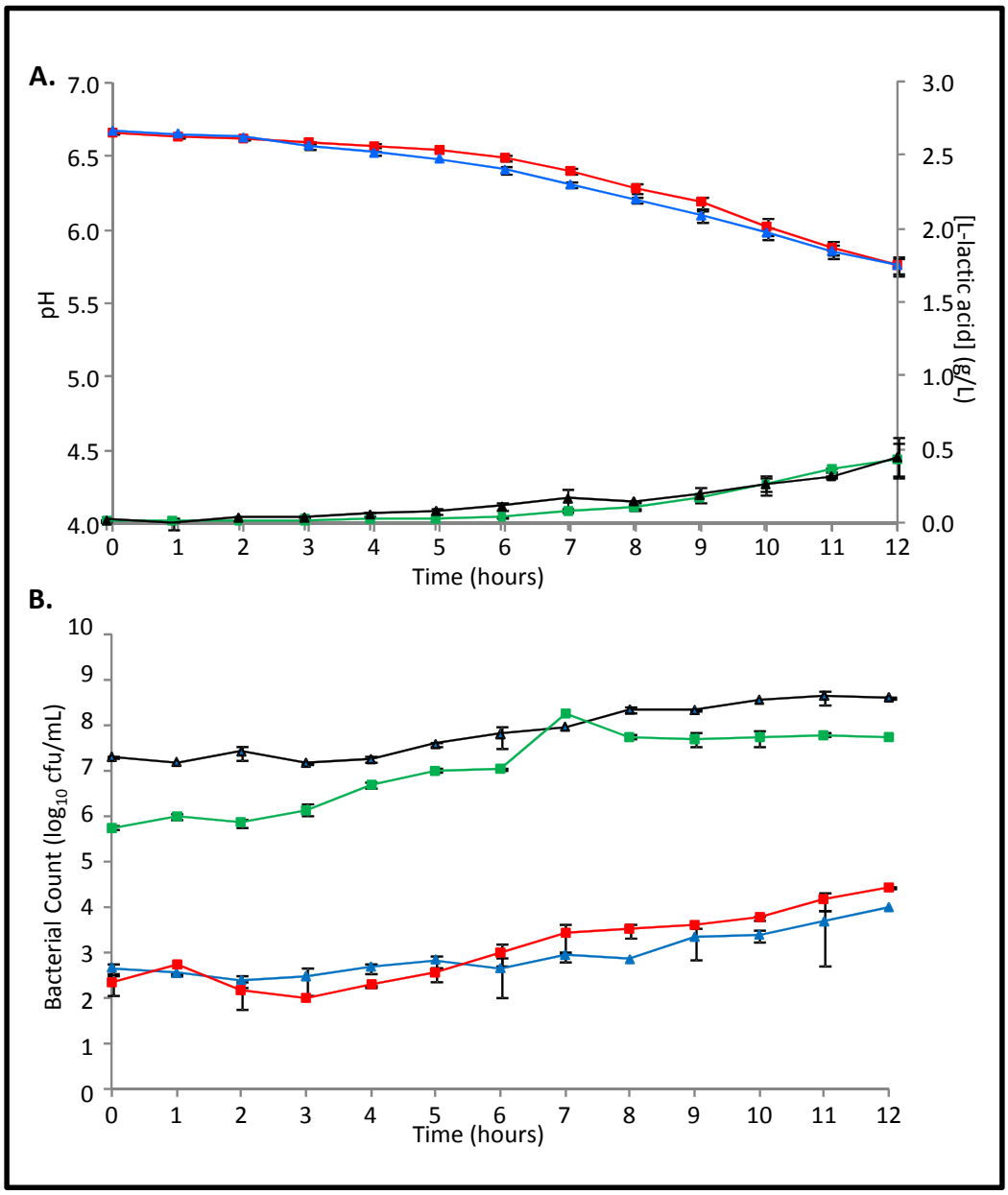

Figure 12. Effect of cheese starter culture B on growth and survival of a low *S. aureus* inoculum. Assays were performed in matched pasteurised and raw milks.

A. pH and L-lactic acid concentration

- Raw Milk – pH
- Pasteurised Milk - pH
- Raw Milk – L-lactic acid concentration
- Pasteurised Milk – L-lactic acid concentration

B. Bacterial growth during 12-hour milk fermentation

- Raw Milk – Starter culture
- Pasteurised Milk – Starter culture
- Raw Milk – *S. aureus*
- Pasteurised Milk – *S. aureus*

Data is presented as the average of duplicate milk aliquots. Error bars are ±1 standard deviation.

4.1.3 Summary

In summary, both challenge trials *S. aureus* numbers were observed to increase regardless of milk type, decreased pH, lactic acid concentration or starter culture used. In all cases a lag period was observed to occur prior to the cell numbers increasing, which was in general shorter in pasteurised milk compared to raw milk, suggesting that there may be some competition or inhibition arising from the raw milk microflora or a component of raw milk that is lost during processing.

4.2 *Listeria monocytogenes* Challenge Trials 1 and 2

In total, four independent milks were used for the Listeria challenge trials. *Listeria* challenge trial one was carried out over two days using two independent matched raw and pasteurised milks. The second challenge trial was also carried out over two days using independent milk samples. Milk composition for each of the challenge trials is listed in Table 3. In all cases, β-lactam antibiotic testing of initial milk samples showed no detectable antibiotics that would inhibit either starter culture or pathogen growth.

Table 3. Milk composition for *L. monocytogenes* inoculum challenge trials

Component	Percentage* (%)			
	Challenge Trial 1 - HI	Challenge Trial 1 - LI**	Challenge Trial 2 - HI	Challenge Trial 2 - LI
Fat	4.73	5.06/4.08	4.6	5.38
Protein	3.61	3.65/3.66	3.54	3.78
Lactose	4.69	4.76/4.79	4.75	4.78
Total Solids	13.62	14.05/13.13	13.38	14.54

*Data supplied by Fonterra
**Raw/Pasteurised data supplied

4.2.1 *L. monocytogenes* Challenge Trial 1

Two independent milk samples were obtained for this challenge trial HI and LI (Table 3). Lactic acid bacteria (LABs) were detected in both raw milks ($2.5 \times 10^3 \pm 9.9 \times 10^2$ cfu/mL and $7 \times 10^2 \pm 1.4 \times 10^2$ cfu/mL; day 1 and day 2 respectively). No lactic acid bacteria were detected in the pasteurised milk at the start of either day of the *L. monocytogenes* trials (data not shown). No *Listeria* was recovered from either pasteurised or raw milk.

L. monocytogenes only analysis was carried out on the second day of the *Listeria* challenge trial using a starting inoculum of $2.1 \times 10^6 \pm 3.0 \times 10^5$ cfu/mL and $1.7 \times 10^6 \pm$

3.5 x 10^4 cfu/mL pasteurised and raw milks (Figure 13). Initial pH of raw and pasteurised milk was 6.69 and 6.69 ± 0.01 respectively. No significant change in pH was observed over the 12 hours of incubation for both milk types with no additions or when *L. monocytogenes* was added (Figure 13 – upper graphs). After 12 hours of incubation at 30 °C, the pH of raw and pasteurised milk was 6.66 ± 0.01 and in the presence of *L. monocytogenes,* 6.65 and 6.61 ± 0.05 respectively. No lactic acid was detected in any of these milk aliquots over the 12-hour period (data not shown).

In pasteurised milk, *L. monocytogenes* cell numbers remained the same over the 12 hours of the experiment, however a small but not significant drop was observed for *L. monocytogenes* in raw milk (2.9 x 10^6 and 4.6 x 10^5 cells/mL, p=0.5; Figure 13 – lower graphs).

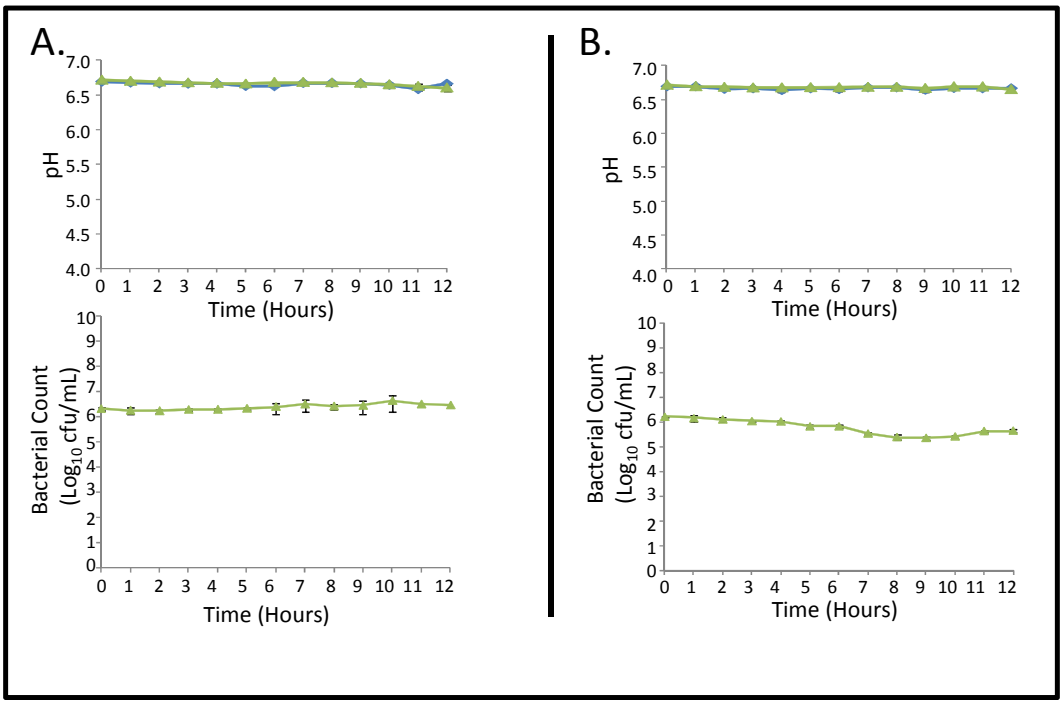

Figure 13. *L. monocytogenes* growth in pasteurised (A) and raw (B) milk at 30 °C.

No addition - Blue diamonds (pH only – Upper graphs).
L. monocytogenes added – Green triangles.
Data is presented as the average of duplicate milk aliquots. Error bars are ±1 standard deviation.

As previously described freeze-dried starter cultures were prepared in peptone water and allowed to fully rehydrate before being added to milk samples as appropriate.

Two inoculum concentrations were used – a high inoculum of 10^5 and a low inoculum of 10^2 cfu/mL. The actual inoculum added was determined at the T = 0 time point. For

the high inoculum, the average final inoculum was 1.9 x 10^6 ± 3.1 x10^5 and 2 x 10^6 ± 5.4 x 10^5 cfu/mL in pasteurised and raw milk respectively. For the low inoculum, the average final inoculum was 3.6 x 10^2 ± 8.7 x 10^1 and 3.7 x 10^2 ± 1.7 x 10^2 cfu/mL in pasteurised and raw milk respectively.

Similar pH and lactic acid data was obtained for the *Listeria* challenge trial as had been observed for the *S. aureus* challenge trials. In summary, in the presence of both starter cultures, pH was observed to decrease after 5 hours of incubation. This pH decrease coincided with an increase in concentration of L-lactic acid (Figure 14A, 15A, 16A and 17A). Starter culture A produced more L-lactic acid than Starter culture B, which resulted in a lower final pH in milk samples containing Starter culture A. However, in raw milk, both starter cultures produced less lactic acid, which resulted in a smaller pH decrease.

For the both the high and low *L. monocytogenes* inoculum, no significant decrease in cell numbers was detected over the 12-hour fermentation, regardless of type of milk or starter culture used (Figure 14B, 15B, 16B and 17B).

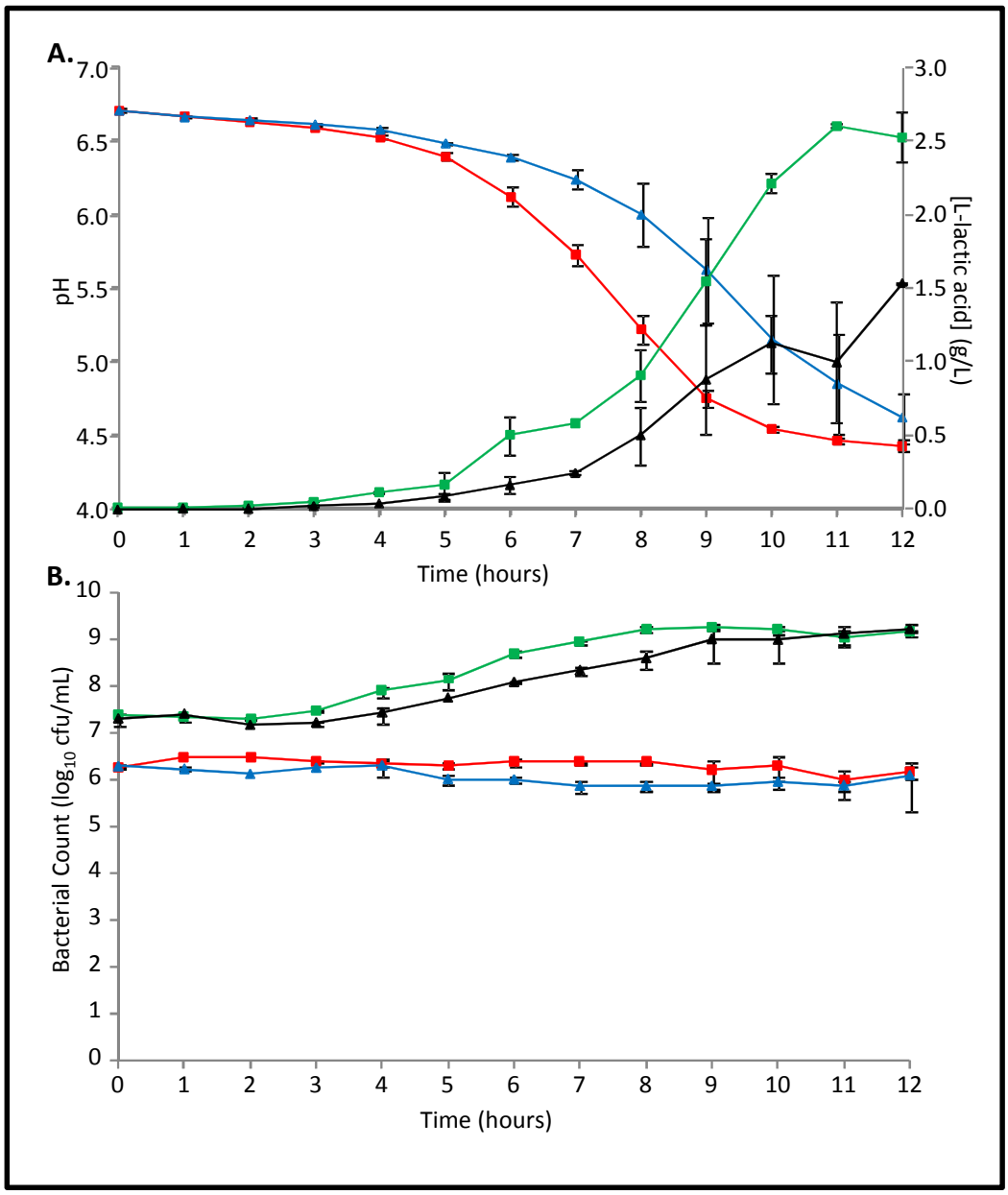

Figure 14. Effect of cheese starter culture A on growth and survival of a high *L. monocytogenes* inoculum. Assays were performed in matched pasteurised and raw milks.

A. pH and L-lactic acid concentration

- Raw Milk – pH
- Pasteurised Milk - pH
- Raw Milk – L-lactic acid concentration
- Pasteurised Milk – L-lactic acid concentration

B. Bacterial growth during 12-hour milk fermentation

- Raw Milk – Starter culture
- Pasteurised Milk – Starter culture
- Raw Milk – *L. monocytogenes*
- Pasteurised Milk – *L. monocytogenes*

Data is presented as the average of duplicate milk aliquots. Error bars are ±1 standard deviation.

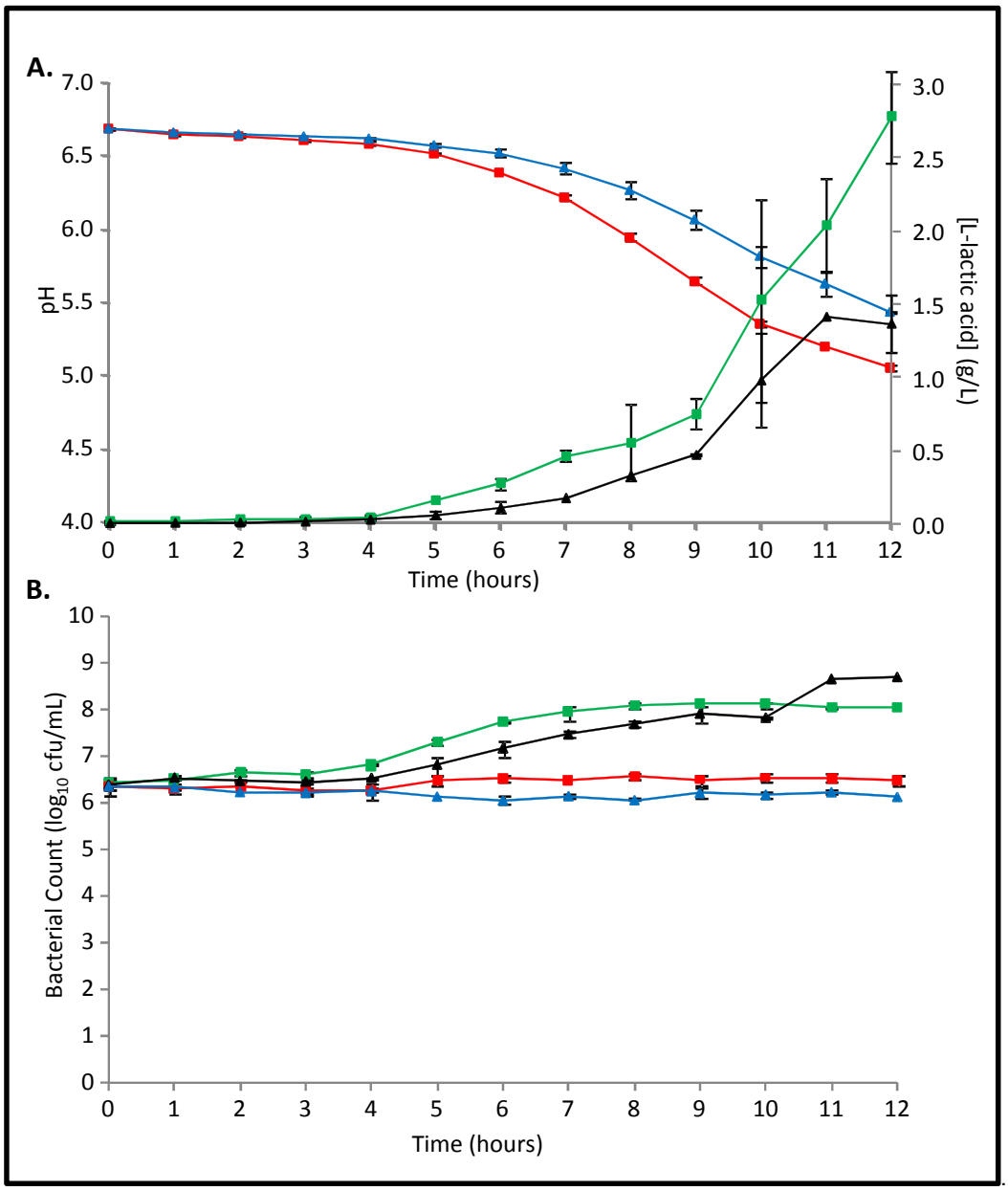

Figure 15. Effect of cheese starter culture B on growth and survival of a high *L. monocytogenes* inoculum. Assays were performed in matched pasteurised and raw milks.

A. pH and L-lactic acid concentration

- Raw Milk – pH
- Pasteurised Milk - pH
- Raw Milk – L-lactic acid concentration
- Pasteurised Milk – L-lactic acid concentration

B. Bacterial growth during 12-hour milk fermentation

- Raw Milk – Starter culture
- Pasteurised Milk – Starter culture
- Raw Milk – *L. monocytogenes*
- Pasteurised Milk – *L. monocytogenes*

Data is presented as the average of duplicate milk aliquots. Error bars are ±1 standard deviation.

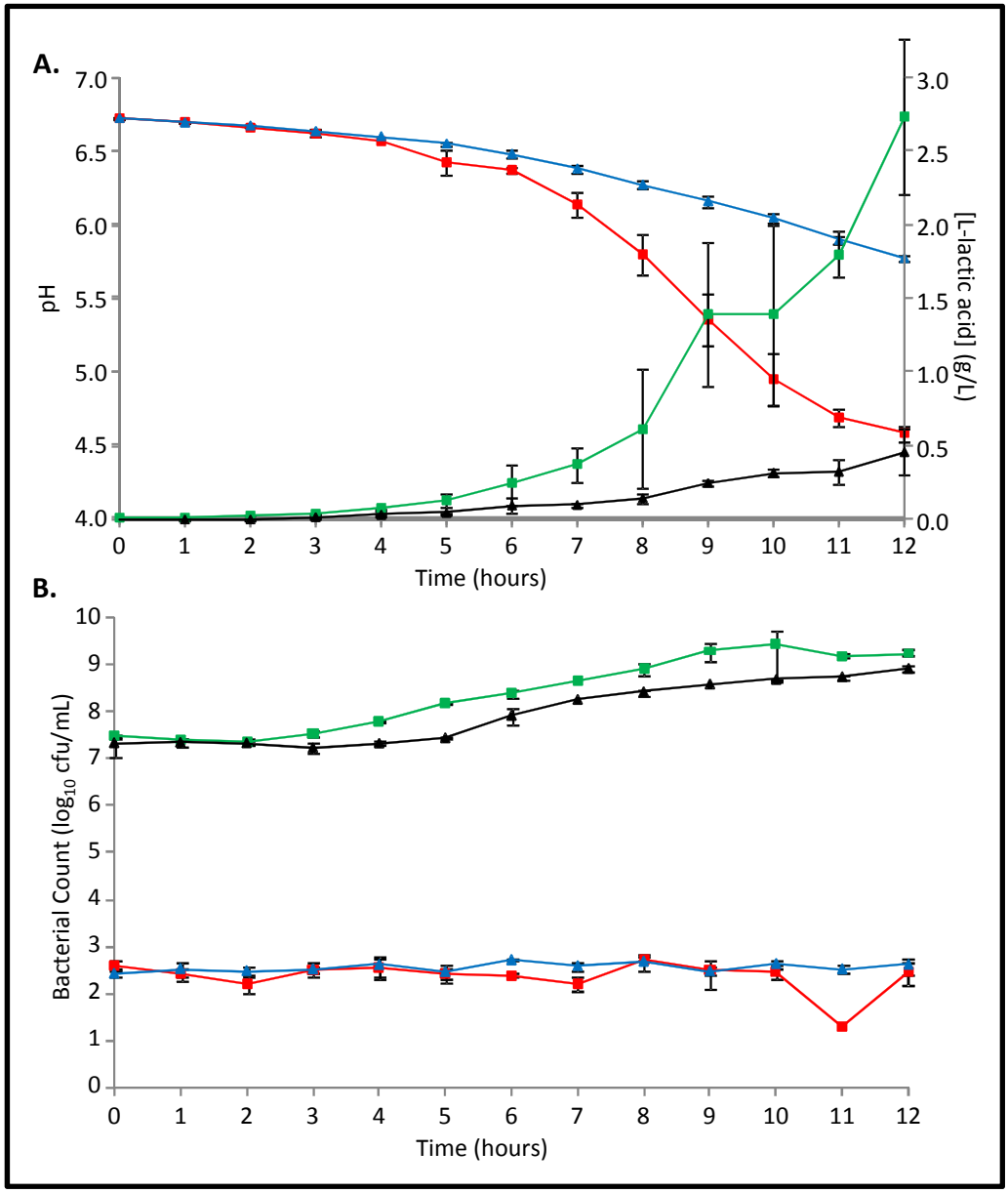

Figure 16. Effect of cheese starter culture A on growth and survival of a low *L. monocytogenes* inoculum.

Assays were performed in matched pasteurised and raw milks.

A. pH and L-lactic acid concentration

- Raw Milk – pH
- Pasteurised Milk - pH
- Raw Milk – L-lactic acid concentration
- Pasteurised Milk – L-lactic acid concentration

B. Bacterial growth during 12-hour milk fermentation

- Raw Milk – Starter culture
- Pasteurised Milk – Starter culture
- Raw Milk – *L. monocytogenes*
- Pasteurised Milk – *L. monocytogenes*

Data is presented as the average of duplicate milk aliquots. Error bars are ±1 standard deviation.

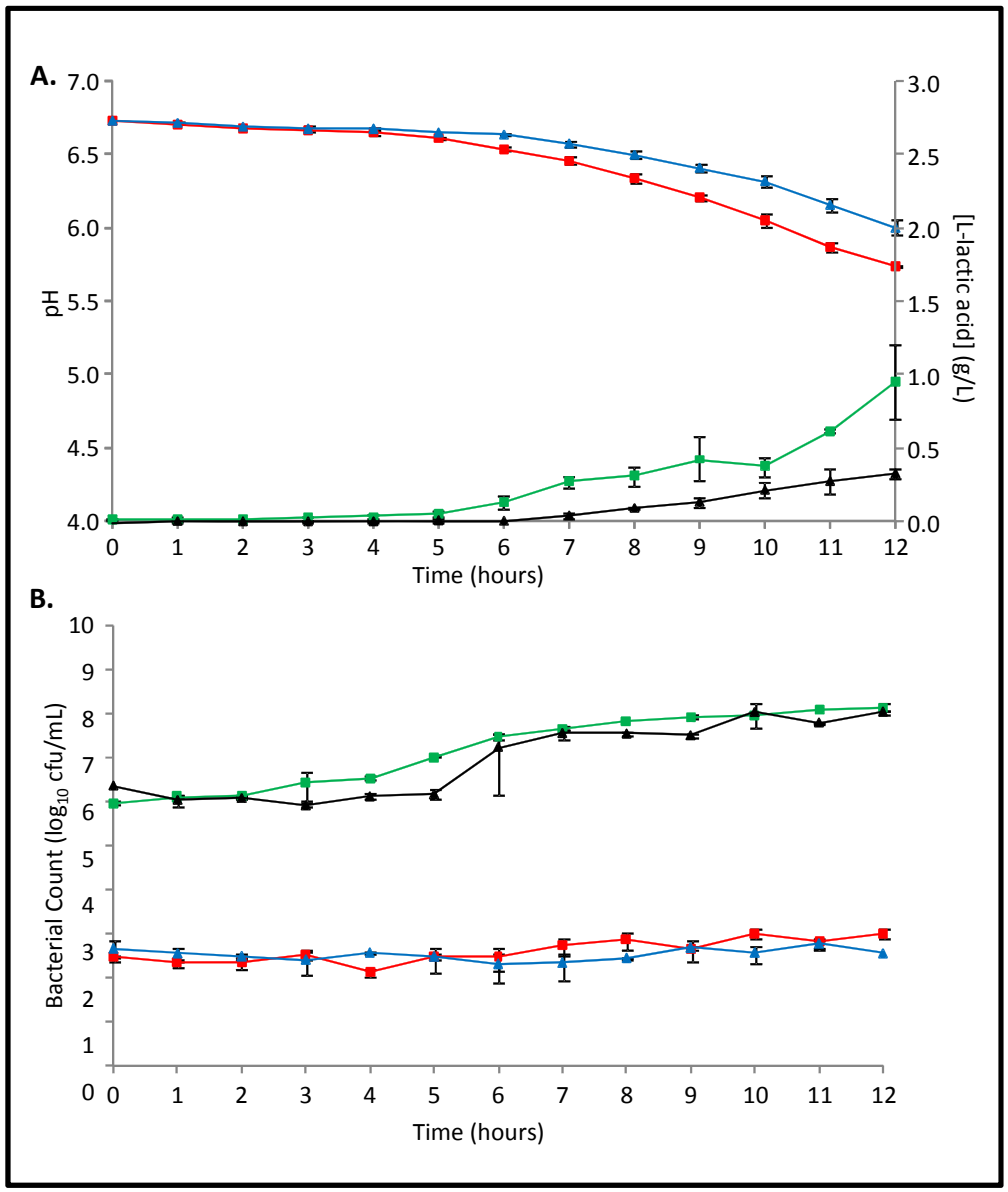

Figure 17. Effect of cheese starter culture B on growth and survival of a low *L. monocytogenes* inoculum.

Assays were performed in matched pasteurised and raw milks.

A. pH and L-lactic acid concentration

- Raw Milk – pH
- Pasteurised Milk - pH
- Raw Milk – L-lactic acid concentration
- Pasteurised Milk – L-lactic acid concentration

B. Bacterial growth during 12-hour milk fermentation

- Raw Milk – Starter culture
- Pasteurised Milk – Starter culture
- Raw Milk – *L. monocytogenes*
- Pasteurised Milk – *L. monocytogenes*

Data is presented as the average of duplicate milk aliquots. Error bars are ±1 standard deviation.

4.2.2 *L. monocytogenes* Challenge Trial 2

Two independent milk samples were obtained from Fonterra Pilot Plant for this challenge trial, HI and LI (Table 3). Lactic acid bacteria (LABs) were detected in both raw milks used in trial 2 ($1.1 \times 10^4 \pm 3 \times 10^3$ cfu/mL and $2.2 \times 10^3 \pm 1.5 \times 10^2$ cfu/mL HI and LI respectively). No lactic acid bacteria were detected in the pasteurised milk at the start of both high and low *L. monocytogenes* trials (data not shown). No *Listeria* was recovered from either pasteurised or raw milk.

L. monocytogenes only analysis was carried out on the second day of the *Listeria* challenge trial in LI milk samples (Figure 18). Initial pH of both raw and pasteurised milk was 6.66 and 6.64 ± 0.02 respectively. No significant change in pH was observed over the 12-hours incubation period for both milk types with either no addition or when only *L. monocytogenes* was added (Figure 18 A and B – upper graphs). After 12 hours at 30 °C, the pH of raw and pasteurised milk was 6.66 ± 0.01 and in the presence of *L. monocytogenes,* 6.65 and 6.61 ± 0.05 respectively. No lactic acid was detected in any of these milk aliquots over the 12-hour fermentation (data not shown).

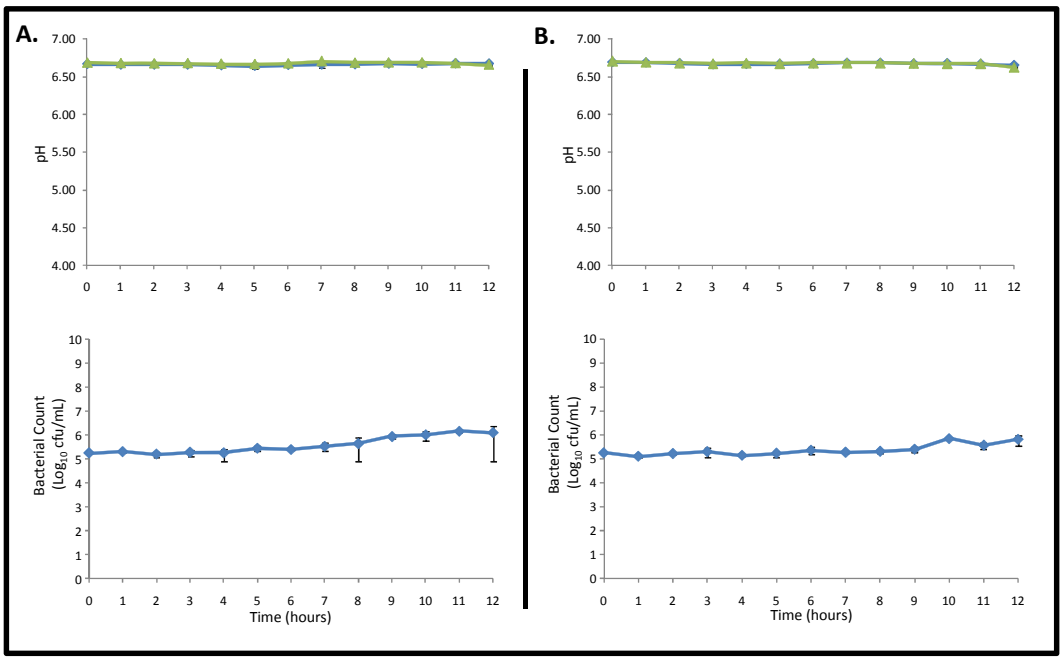

Figure 18. *L. monocytogenes* growth in pasteurised (A) and raw (B) milk at 30 °C.

No addition control - Green triangles.
L. monocytogenes added – Blue diamonds.
Data is presented as the average of duplicate milk aliquots. Error bars are ±1 standard deviation.

L. monocytogenes was added to both pasteurised and raw milk with a starting inoculum of $1.8 \times 10^5 \pm 5.2 \times 10^3$ cells/mL to determine pathogen growth or survival in milk. In both milk types, *L. monocytogenes* cell numbers increased by approximately 1

log over the 12 hours of the experiment regardless of milk type (observed final concentration 1.4 x 10^6 ± 1.1 x 10^6 and 6.8 x 10^5 ± 3.1 x 10^5 cells/mL for pasteurised and raw milks respectively; Figure 18 A and B – lower graphs).

As previously described freeze-dried starter cultures were prepared in peptone water and allowed to fully rehydrate before being added to milk samples as appropriate.

Two inoculum concentrations were used in the presence of two starter cultures – a high inoculum of 10^5 and a low inoculum of 10^2 cfu/mL. The final inoculum of *Listeria* added was determined at the T = 0 time point. For the high inoculum, the average inital inoculum was 1.8 x 10^5 ± 1.9 x10^4 and 1.8 x 10^5 ± 3.3 x 10^4 cfu/mL in pasteurised and raw milk respectively. For the low inoculum, the average final inoculum was 1.1 x 10^2 ± 9.0 x 10^1 and 6.0 x 10^2 ± 2.2 x 10^2 cfu/mL in pasteurised and raw milk respectively.

Similar pH and lactic acid data profiles were obtained for the second *Listeria* challenge trial as had been observed for the first challenge trial. In summary, in the presence of both starter cultures, pH was observed to decrease after 5 hours of incubation. This pH decrease coincided with an increase in concentration of lactic acid (Figures 19A - 22A). Starter culture A produced more lactic acid than Starter B, which resulted in a lower final pH in milk samples containing Starter culture A. However, in raw milk, both starter cultures produced less lactic acid, which resulted in a smaller pH decrease.

For the both the high and low *L. monocytogenes* inoculum, no significant change in cell numbers was detected over the 12-hour fermentation, regardless of type of milk or starter culture used (Figures 19B -22B).

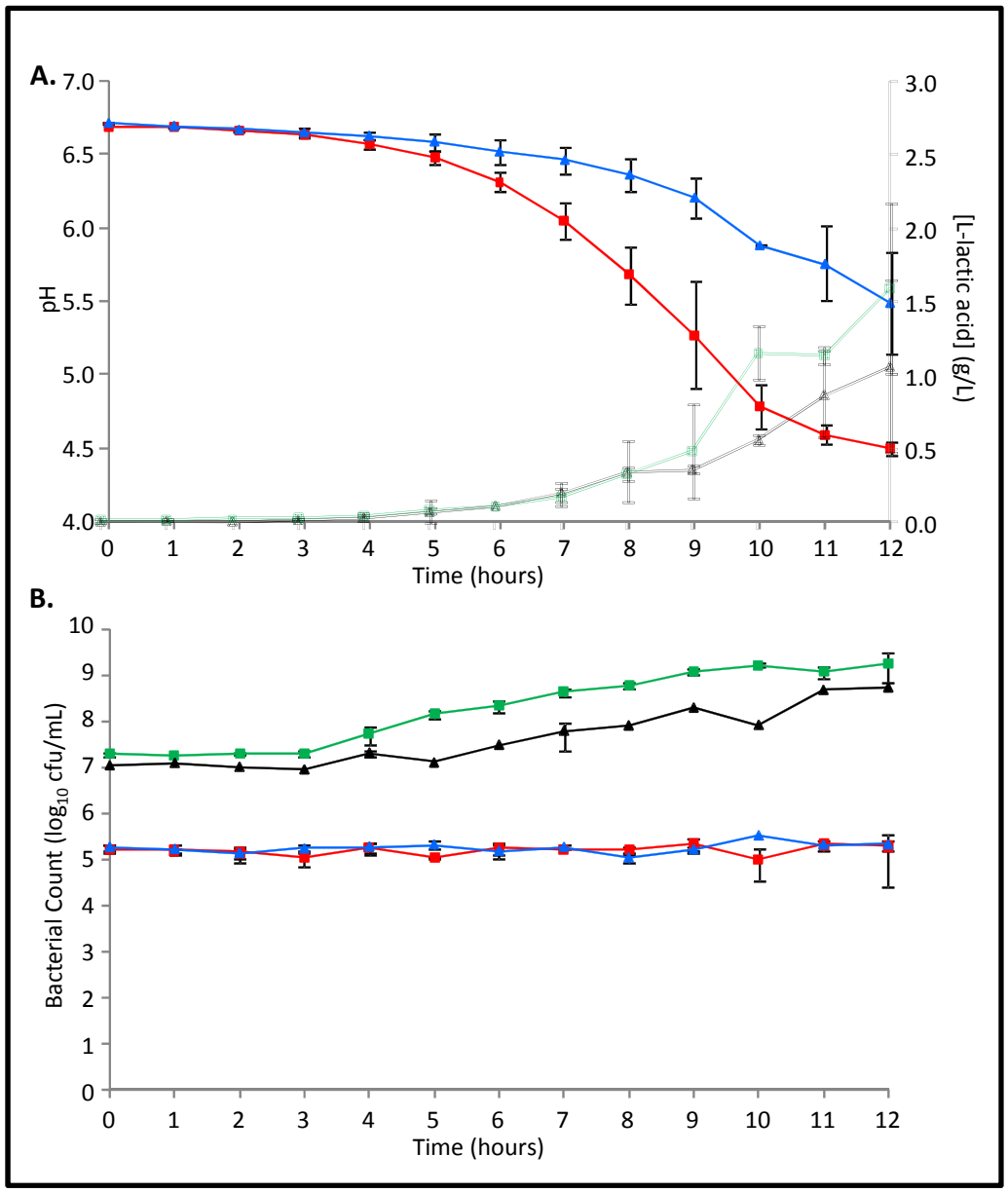

Figure 19. Effect of cheese starter culture A on growth and survival of a high *L. monocytogenes* inoculum. Assays were performed in matched pasteurised and raw milks.

A. pH and L-lactic acid concentration

- Raw Milk – pH
- Pasteurised Milk - pH
- Raw Milk – L-lactic acid concentration
- Pasteurised Milk – L-lactic acid concentration

B. Bacterial growth during 12-hour milk fermentation

- Raw Milk – Starter culture
- Pasteurised Milk – Starter culture
- Raw Milk – *L. monocytogenes*
- Pasteurised Milk – *L. monocytogenes*

Data is presented as the average of duplicate milk aliquots. Error bars are ±1 standard deviation.

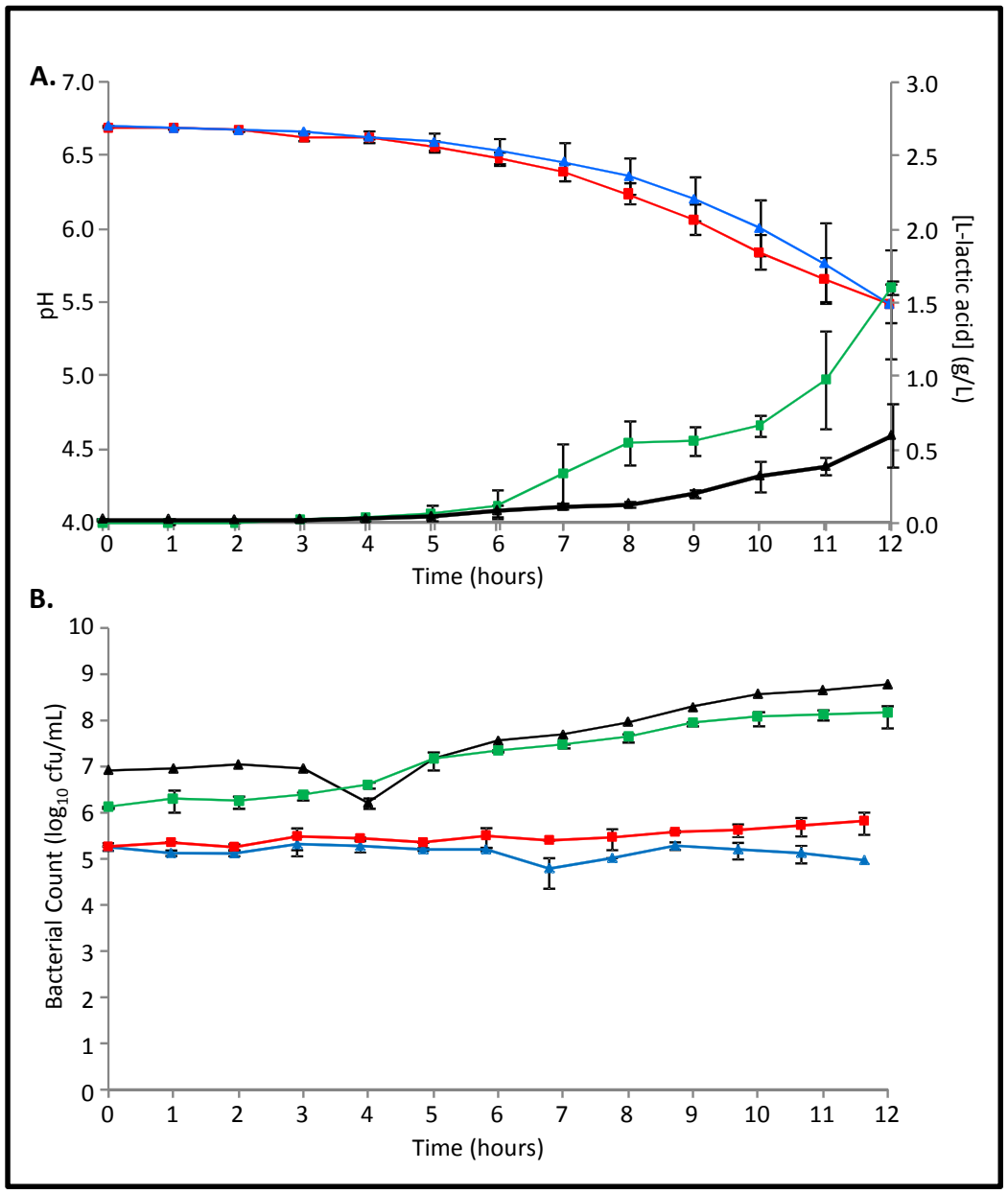

Figure 20. Effect of cheese starter culture B on growth and survival of a high *L. monocytogenes* inoculum. Assays were performed in matched pasteurised and raw milks.

A. pH and L-lactic acid concentration

- Raw Milk – pH
- Pasteurised Milk - pH
- Raw Milk – L-lactic acid concentration
- Pasteurised Milk – L-lactic acid concentration

B. Bacterial growth during 12-hour milk fermentation

- Raw Milk – Starter culture
- Pasteurised Milk – Starter culture
- Raw Milk – *L. monocytogenes*
- Pasteurised Milk – *L. monocytogenes*

Data is presented as the average of duplicate milk aliquots. Error bars are ±1 standard deviation.

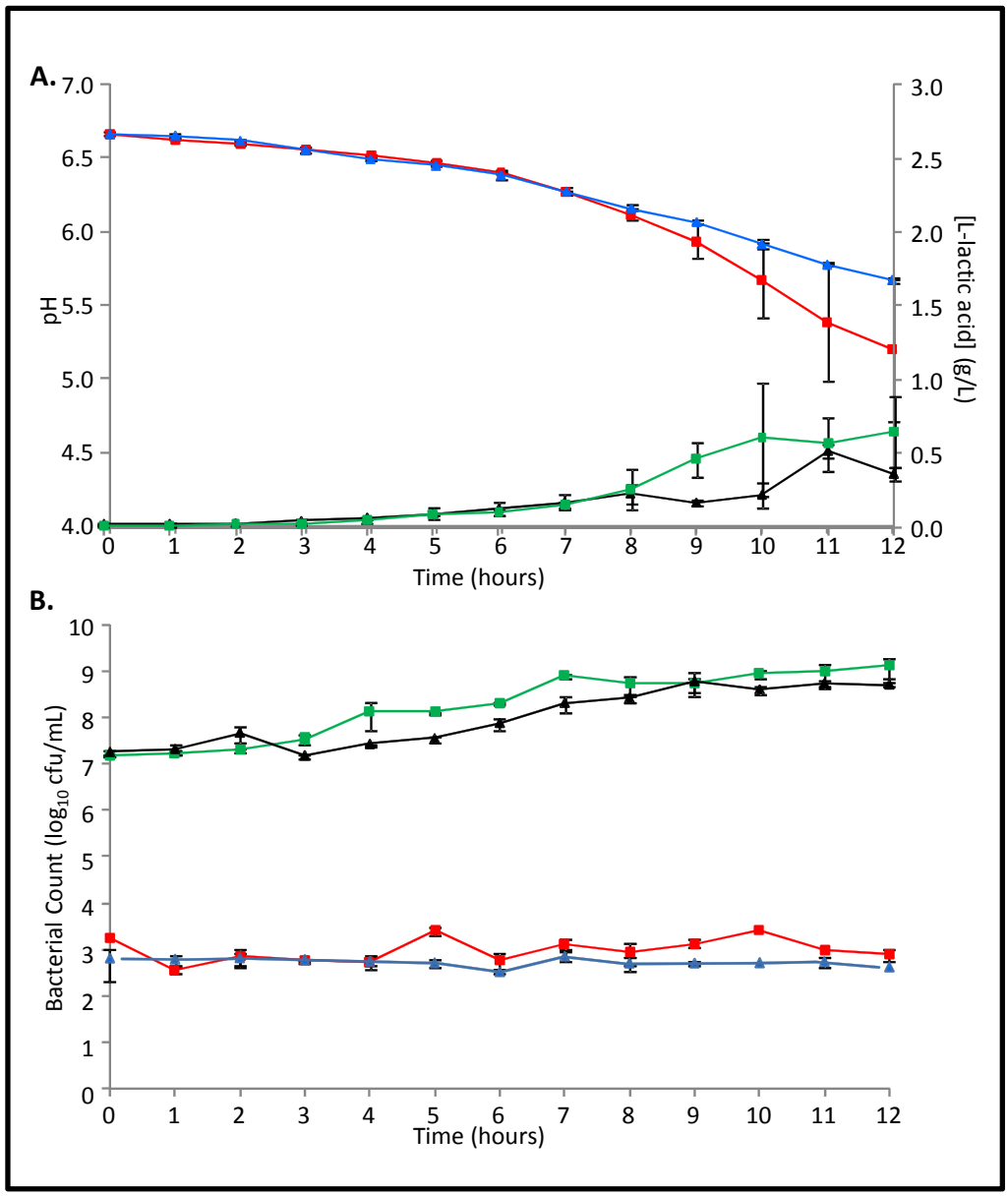

Figure 21. Effect of cheese starter culture A on growth and survival of a low *L. monocytogenes* inoculum.

Assays were performed in matched pasteurised and raw milks.

A. pH and L-lactic acid concentration

- Raw Milk – pH
- Pasteurised Milk - pH
- Raw Milk – L-lactic acid concentration
- Pasteurised Milk – L-lactic acid concentration

B. Bacterial growth during 12-hour milk fermentation

- Raw Milk – Starter culture
- Pasteurised Milk – Starter culture
- Raw Milk – *L. monocytogenes*
- Pasteurised Milk – *L. monocytogenes*

Data is presented as the average of duplicate milk aliquots. Error bars are ±1 standard deviation.

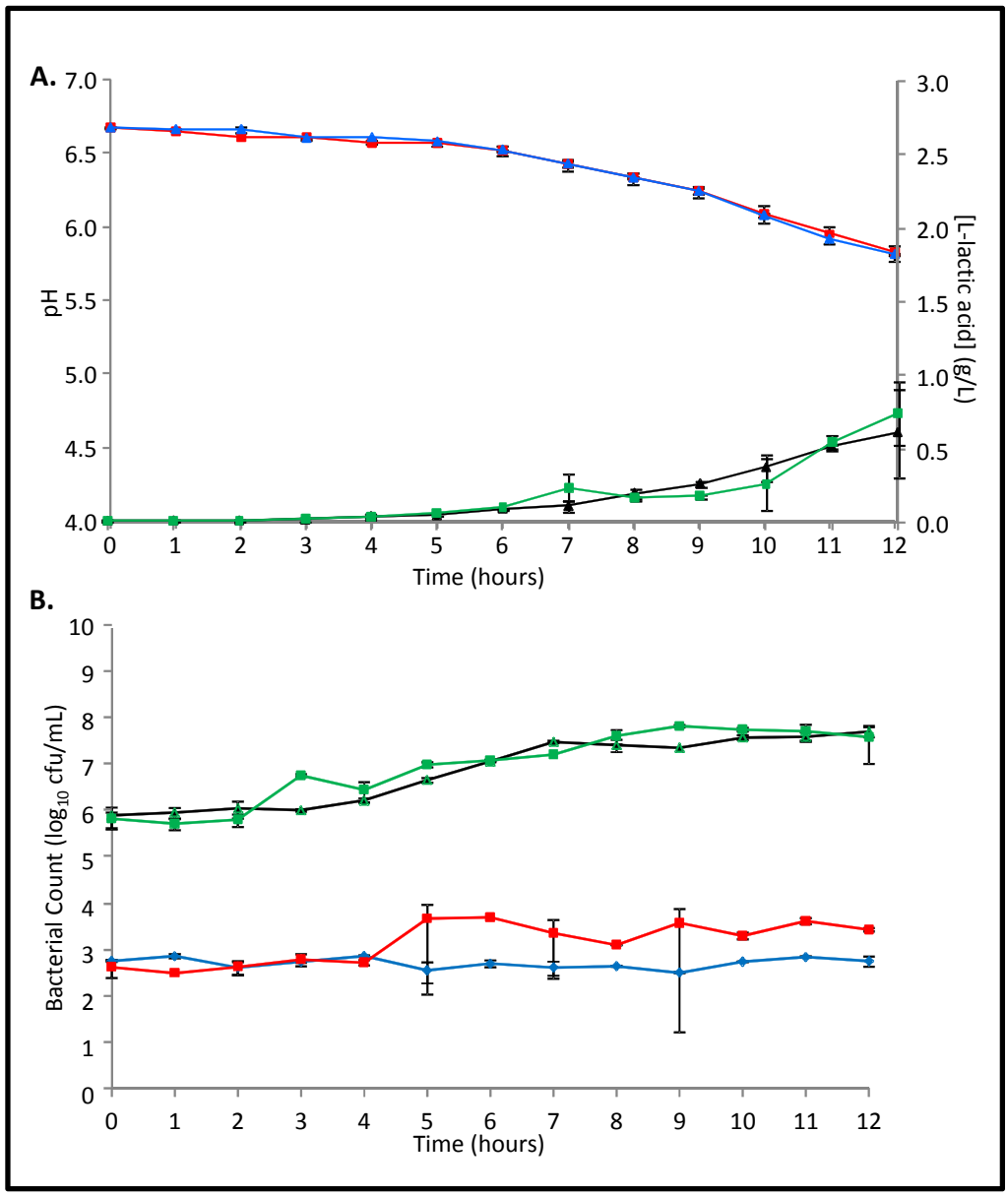

Figure 22. Effect of cheese starter culture B on growth and survival of a low *L. monocytogenes* inoculum.

Assays were performed in matched pasteurised and raw milks.

A. pH and L-lactic acid concentration

- Raw Milk – pH
- Pasteurised Milk - pH
- Raw Milk – L-lactic acid concentration
- Pasteurised Milk – L-lactic acid concentration

B. Bacterial growth during 12-hour milk fermentation

- Raw Milk – Starter culture
- Pasteurised Milk – Starter culture
- Raw Milk – *L. monocytogenes*
- Pasteurised Milk – *L. monocytogenes*

Data is presented as the average of duplicate milk aliquots. Error bars are ±1 standard deviation.

4.2.3 Summary

In summary, for both *L. monocytogenes* challenge trials no significant change in *L. monocytogenes* numbers were observed regardless of the milk type, pH change, lactic acid concentration or starter culture used.

4.3 Lactose Assays

Lactose assays were performed on milk samples that had been treated to remove the casein. Controls used in these assays were samples of milk with associated data from Fonterra, and 1.47 – 560 mg/mL lactose solutions to form a standard curve. The standard curves calibrating the assay worked well with a reasonable degree of reproducibility. However, the milk associated data generated did not agree with data supplied by Fonterra and reproducibility was poor. Sample preparation modifications have been tried without success.

5. Discussion

These challenge trials were undertaken to examine whether two important food borne pathogens, *Listeria monocytogenes* and *Staphylococcus aureus*, can survive or grow in raw milk during fermentation by starter cultures used in commercial cheese manufacture. Comparison between matched raw and pasteurised milk was made to determine whether or not the naturally occurring milk microflora present in raw milk would impact on their survival. Parameters used during these trials were based on data generated in the pilot trial. It is worth noting that initial sampling of the raw milk from both trials revealed that *L. monocytogenes* was not present in either raw milk samples while *S. aureus* was present in raw milk.

Cheese manufacture is a complex process that involves many different stages that can affect microbial viability (Estrada et al, 1999; Schvartzman et al, 2011; Linton et al, 2008). Although there are many different cheeses available, there are some consistent key steps in most cheese manufacturing processes. These include initial fermentation (which produces lactic acid resulting in the initial decrease in pH), renneting, scalding, salt addition and water reduction. Most continental style cheeses, after pressing, have a pH of between 5.3 and 5.7 with a final pH after 24 hours of between 5.1 and 5.3 (depending on the style of cheese manufacture). Once the curd is scalded and pressed, water activity and salt tolerance will ultimately influence microbial survival, particularly of

pathogens. This project focussed solely on liquid milk fermentation which was extended out to 12 hours.

To eliminate any milk composition variables, matched raw and pasteurised milk were sourced locally from the pilot processing plant at Fonterra, Grasslands for this trial. Milk arrives at the pilot plant daily by tanker, where it is processed for use the same day. For these challenge trials, milk was obtained from the pilot plant in the morning and either used the same day or stored at +4 °C for use the following day. Trial milk was used within 24 hours of pasteurisation. It should be noted that the process of pasteurisation alters the chemical components within the milk that may support microbial growth, by increasing the availability of simple sugars for fermentation. For *S. aureus* Trial 1, the high inoculum trial was carried out the same day and the low inoculum trial carried out the following day within the prescribed 24 hours using the same milk. *S. aureus* Trial 2 used different milks for each of the high and low inoculum challenge trials. Both the *L. monocytogenes* trials were carried out the following day after pasteurisation, each trial used a different milk set.

Two different starter cultures were selected for this trial; both of which are used in the manufacture of hard continental style cheeses. Although both are primarily composed of similar *Lactococcus* species*,* starter culture B (FD-DVS FLORA-DANICA) also contains *Leuconostoc mesenteroides* subsp *cremoris.* Optimal growth temperature to maximise pH reduction is 30 °C, with lowest pH levels of approximately 4.5 achieved after fermentation of 10 hours and 12 hours for starter A and starter B respectively (Chr Hansen, 2001a; 2001b). This is considerably longer than most commercial fermentation processes before the addition of either rennet and/or salt. Continual pH decreases occur as the curd is formed. The appearance of curd occurred in these trials when the pH dropped below 5.0 regardless of the type of milk used. Although lactic acid occurs in two isoforms, D and L, only L-lactic acid (the biologically active form of lactic acid), was detected during milk fermentation. The range of end products produced during fermentation of carbohydrates within the milk, i.e. from homo or hetero-fermentation, depends upon bacterial species found in the starter culture (Carr et al 2002).

To reflect the NZ situation, 6 isolates of each pathogen were obtained from Fonterra's culture collection of bacteria isolated from milk or milk products (Table 1). Analysis of pH sensitivity and optimal growth conditions were carried out on each of these bacterial isolates, and confirmed that these isolates were within the expected range of

growth parameters (data not shown). pH ranges for growth of type strains of *Staphylococcus* are pH 4.2 to pH 9.3 and pH 4.4 to pH 9.4 for *Listeria.* All isolates used in this study, both *Listeria* and *Staphylococcus,* were able to survive and grow at pH 4.5 in laboratory broth assays. Given this, it is therefore not surprising that both *Staphylococcus* and *Listeria* survived milk fermentation where the lowest pH achieved was pH 4.43. These low pHs were only achieved after a minimum of 9 hours fermentation and therefore the exposure time at these pHs was not long during this trial. In addition, it is likely that the gradual decrease in pH will have allowed significant bacterial adaptation to decreasing pH to occur, increasing the likelihood of survival. *S. aureus* was able to grow in both pasteurised and raw milks.

There was a difference in the observed lag time with growth being observed earlier in pasteurised milk compared to raw milk. This difference is likely to be attributed to the higher availability of simple sugars or nutrients to support the growth of *S. aureus* in pasteurised compared to raw milk. *Listeria* numbers did not significantly increase in either the presence or absence of starter cultures. Marshall et al. (1991) observed that not all *Listeria monocytogenes* isolates were capable of using lactose as a primary carbon source, however they were capable of growing in milk were partial hydrolysis of proteins had occurred. Similar studies by Pine et al (1989) confirmed these findings, suggesting that only the glucose moiety of lactose was used by *L. monocytogenes*. Generation times of *L. monocytogenes* in processed milk can vary widely depending on the level of chemical modification that has occurred as well as the incubation temperatures used. This may in part explain why no *Listeria* growth was observed in this trial.

6. Conclusions

The main conclusion from this study was that the pH changes that occur during the milk fermentation stage with the increasing concentration of L-lactic acid are not sufficient to eliminate or inhibit the growth of either *Listeria* or *Staphylococcus.* Most importantly, pH values likely to influence survival of these pathogens do not occur till very late in milk fermentation, if at all and are not likely to be achieved during normal fermented product manufacture. It is likely that changes in pH and L-lactic acid over time in conjunction with other stages of processing may act in concert as critical control points.

Naturally-occurring milk flora present in raw milk did not influence starter culture survival or pathogen survival, although the achieved pH and lactic acid concentrations were adversely affected in raw milk.

From data presented here, it is clear that raw milk contaminated with either *Staphylococcus aureus* or *Listeria monocytogenes* should not be used in the manufacture of a raw milk fermented product where pH or lactic acid are the primary pathogen critical control points.

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7. References

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8. Appendices

8.1 Appendix 1 – Starter culture performance data sheets

CHR. HANSEN

FD-DVS FLORA-DANICA

Product Information

Description

Mesophilic Aromatic Culture, type LD.
Multiple mixed strain culture containing *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris* and *Lactococcus lactis* subsp. *diacetylactis*. The culture produces flavor and CO_2.

FLORA-DANICA is packed in a convenient freeze-dried form.

Application

The culture is primarily used in the manufacture of Continental cheese types (Gouda, Edam, Leerdam, Samsoe) and soft cheese types (Lactic cheeses, Camembert, Blue cheese).

Packing

Packing size	Item number
10 X 50U	100103
25 X 200U	100129
20 X 500U	100163

Availability

In addition to FLORA-DANICA, other cultures in this series include CHN-11, CHN-19, CHN-120 and B-11.

Storage and shelf life

Freeze-dried cultures should be stored at -18°C (0°F) or below. If the cultures are stored at -18°C (0°F) or below, the shelf life is at least 24 months. At +5°C (41°F) the shelf life is at least 6 weeks.

Instructions for use

Remove the cultures from the freezer just prior to use. **DO NOT THAW THESE CULTURES.** Sanitize the top of the pouch with chlorine. Open the pouch and pour the freeze-dried granules directly into the pasteurized product using slow agitation. Agitate the mixture for 10-15 minutes to distribute the culture evenly.

Dosage

Recommended dosage of freeze-dried DVS cultures in units to liters:

DVS inoculation percentage	Amount of milk to be inoculated			
	1,000 l	5,000 l	10,000 l	15,000 l
1000U/5000 l	200U	1000U	2000U	3000U
500U/5000 l	100U	500U	1000U	1500U
250U/5000 l	50U	250U	500U	750U

FD-DVS FI-Dan PI EN vs2 Apr2005/1:3

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FD-DVS FLORA-DANICA

Product Information

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Recommended dosage of freeze-dried cultures in units to US lbs:

DVS inoculation percentage	Amount of milk to be inoculated			
	2,270 lbs	11,350 lbs	22,700 lbs	34,000 lbs
1000U/11,350 lbs	200U	1000U	2000U	3000U
500U/11,350 lbs	100U	500U	1000U	1500U
250U/11,350 lbs	50U	250U	500U	750U

As a principal rule 1000U of freeze-dried DVS culture will correspond to 100 l of active bulk starter. However, specific usage rates should be determined experimentally before a new application.

Incubation temperature Recommended incubation temperature is 22-30°C (95-113°F). For more information please use Chr. Hansen's suggested recipes.

Kosher status FLORA-DANICA is Kosher approved (Circle K D) for year-round use, excluding Passover.

Technical Information

- Flavor and gas production

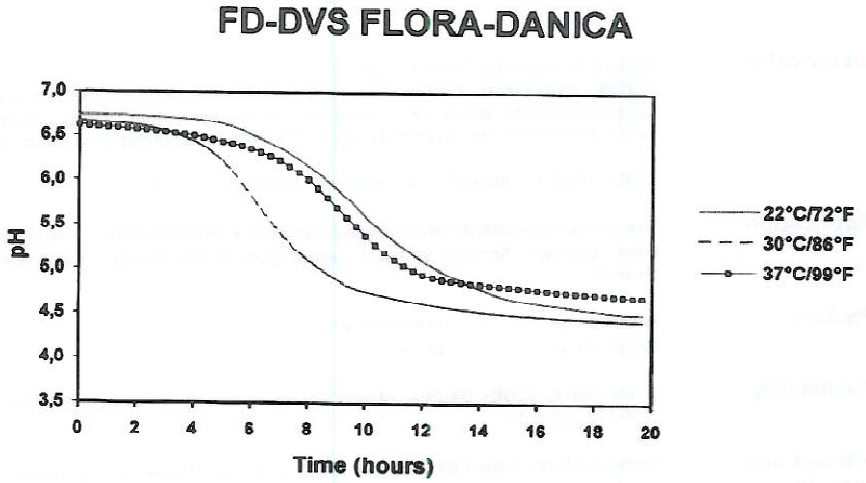
	Flavor:	High
	Gas:	High (CO_2)
- Salt sensitivity	50% inhibition:	3.7% NaCl
	100% inhibition:	6.0% NaCl

FD-DVS Fl-Dan PI EN vs2 Apr2005/2:3

FD-DVS FLORA-DANICA
Product Information

CHR. HANSEN

Figure 1. The effect of temperature on acidification



Fermentation conditions:
Lab milk 9.5% T.S.: 140°C/8 sec. - 100°C/30 min
500U/5000 l inoculation

NB: Note that the accuracy of these curves is relative and subject to experimental error.

Technical service Chr. Hansen's world-wide facilities and the personnel of our application and technology center are at your disposal with assistance and instruction.

References

References and analytical methods are available upon request.

FD-DVS Fl-Dan PI EN vs2 Apr2005/3:3

CHR. HANSEN

F-DVS FLORA-DANICA

Product Information

Description

Mesophilic Aromatic Culture, type LD.
Multiple mixed strain culture containing *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris* and *Lactococcus lactis* subsp. *diacetylactis*. The culture produces flavor and CO_2.

FLORA-DANICA is packed in a convenient frozen pellet form.

Application

The culture is primarily used in the manufacture of Continental cheese types (Gouda, Edam, Leerdam, Samsoe) and soft cheese types (Lactic cheeses, Camembert, Blue cheese).

Packing

Packing size	Item number
500 g carton	501691

Availability

In addition to FLORA-DANICA, other cultures in this series include CHN-11, CHN-19, CHN-120 and B-11.

Storage and shelf life

Frozen cultures should be stored at -45°C (-49°F) or below. If the cultures are stored at -45°C (-49°F) or below, the shelf life is at least 12 months.

Instructions for use

Remove cultures from the freezer just prior to use. **DO NOT THAW THESE CULTURES.** Sanitize the gable top of the carton with chlorine. Open the carton and pour the frozen pellets directly into the pasteurized product using slow agitation. Agitate the mixture for 10 to 15 minutes to distribute the culture evenly.

Dosage

Recommended dosage of frozen DVS cultures in grams to liters:

DVS inoculation percentage	Amount of milk to be inoculated			
	10,000 l	15,000 l	20,000 l	25,000 l
0.005%	500 g	750 g	1000 g	1250 g
0.010%	1000 g	1500 g	2000 g	2500 g
0.015%	1500 g	2250 g	3000 g	3750 g
0.020%	2000 g	3000 g	4000 g	5000 g

ABr/fl-dan-fro.doc/okt 2001/1:3

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CHR. HANSEN

FD-DVS pHageControl™ R-700 Culture Series

Product Information

Description

Mesophilic Homofermentative Culture, type O.
Chr. Hansen's pHage Control culture system provides phage resistant mesophilic defined strains for continuous DVS (direct vat set) use. These cultures contain special strains of *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* selected for their phage resistance and ability to produce lactic acid quickly and do not produce CO_2. Freeze-dried pHage Control cultures are packed in a convenient freeze-dried form.

Application

The culture is primarily applied in the production of cheeses with a closed texture, eg Cheddar, Feta and Cottage Cheese. The culture can be applied in other fermented dairy products, in combination or not with other lactic cultures.

Availability

Available freeze-dried pHage control cultures include R-703, R-704, R-707 and R-708.

Packing

Description	Item no 10 x 50U	Item no 25 x 200U	Item no 20 x 500U	Item no 10 x 1000U
R-703	100095	100122	100156	
R-704	100096	100123	100157	100200
R-707	100097	100124	100158	100201
R-708	100098	100125	100159	

Storage and shelf life

Freeze-dried cultures should be stored at -18°C (0°F) or below. If the cultures are stored at -18°C (0°F) or below, the shelf life is at least 24 months. At +5°C (41°F) the shelf life is at least 6 weeks.

Instructions for use

Remove the cultures from the freezer just prior to use. **DO NOT THAW THESE CULTURES.** Sanitize the top of the pouch with chlorine. Open the pouch and pour the freeze-dried granules directly into the pasteurized product using slow agitation. Agitate the mixture for 10-15 minutes to distribute the culture evenly.

Dosage

Recommended dosage of freeze-dried DVS cultures in units to liters:

DVS inoculation percentage	Amount of milk to be inoculated			
	1,000 l	5,000 l	10,000 l	15,000 l
1000U/5000 l	200U	1000U	2000U	3000U
500U/5000 l	100U	500U	1000U	1500U
250U/5000 l	50U	125U	500U	750U

ABr/R-700 Series-PI/okt 2001/1:5

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Product Information

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Recommended dosage of freeze-dried cultures in units to US lbs:

DVS inoculation percentage	Amount of milk to be inoculated			
	2,270 lbs	11,350 lbs	22,700 lbs	34,000 lbs
1000U/11,350 lbs	200U	1000U	2000U	3000U
500U/11,350 lbs	100U	500U	1000U	1500U
250U/11,350 lbs	50U	125U	500U	750U

As a principal rule 1000U of freeze-dried DVS culture will correspond to 100 l of active bulk starter. However, specific usage rates should be determined experimentally before a new application.

Incubation temperature

The dosage of this culture system is customized to your individual cheese make procedure. Please contact your local Chr. Hansen representative for more information.

Kosher status

pHage Control cultures are Kosher approved (Circle K D) for year-round use, excluding Passover.

Technical information

- Flavor and gas production

	R-703	R-704	R-707	R-708
Flavor	-	-	-	-
Gas	None	None	None	None

- Salt sensitivity

	R-703	R-704	R-707	R-708
50% inhibition	5.0% NaCl	5.5% NaCl	5.3% NaCl	5.7% NaCl
100% inhibition	>6.0% NaCl	>6.0% NaCl	>5.8% NaCl	>6.0% NaCl

ABr/R-700 Series-PI/okt2001/2:5

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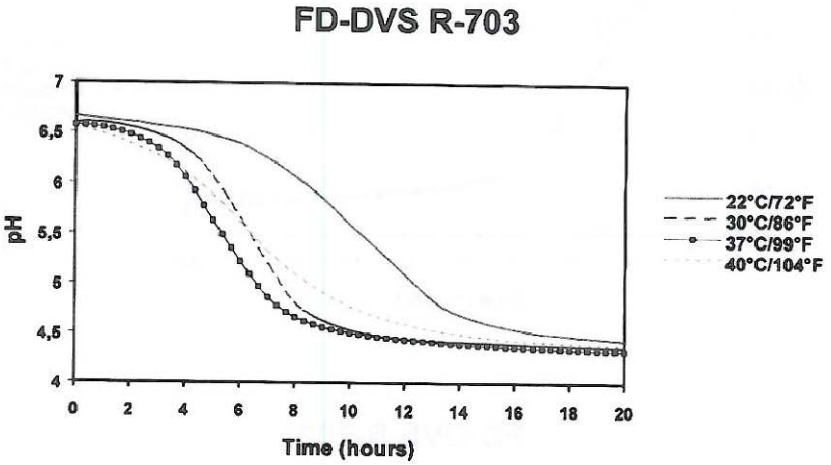
FD-DVS pHageControl™
R-700 Culture Series
Product Information

CHR. HANSEN

Figures 1-4. The effect of temperature on acidification

Fermentation conditions:
Lab milk 9.5% T.S.: 140°C/8 sec. - 100°C/30 min
500U/5000 l inoculation



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Product Information

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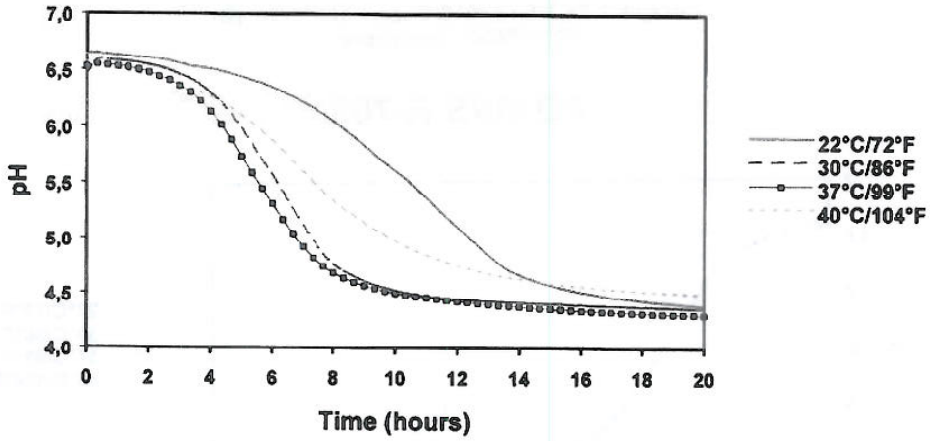
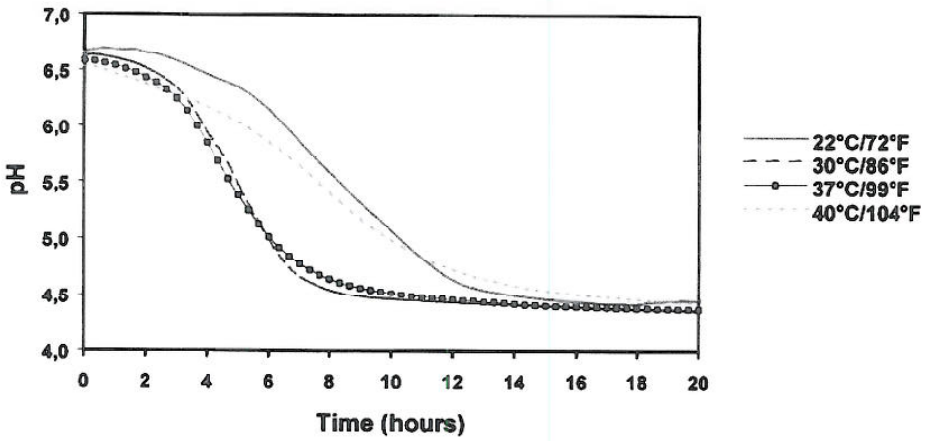
FD-DVS R-704



FD-DVS R-707



ABr/R-700 Series-PI/okt2001/4:5

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FD-DVS pHageControl™ R-700 Culture Series

Product Information

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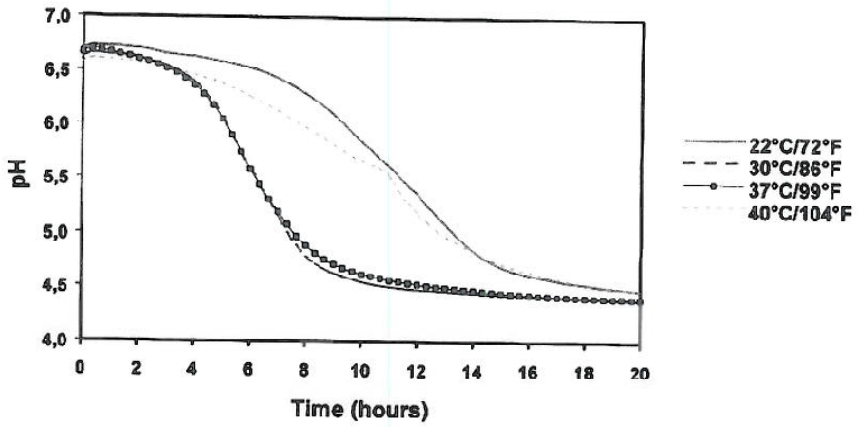
FD-DVS R-708



NB: Note that the accuracy of these curves is relative and subject to experimental error.

Technical service Chr. Hansen's worldwide facilities and the personnel of our application and technology center are at your disposal with assistance and instructions.

References

References and analytical methods are available upon request.

The information contained herein is to our knowledge true and correct and presented in good faith. However, no warranty, guarantee, or freedom from patent infringement is implied or inferred. This information is offered solely for your consideration and verification.

EN-pHage Control-FD-PI-1001

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ABr/R-700 Series-PI/okt2001/5:5

8.2 Appendix 2 – Statistical analysis of challenge trial data

Graphs showing pH values across observed hours, distinguished by type of milk (raw/pasteurised) and challenge trials (T1/T2) are presented in Figure 2.1 for each of the 8 combinations of pathogen (*L. monoytogenes* and *S. aureus*), inoculum (high/low) and starter (A/B) parameters. Similar graphs for lactic acid and bacterial count are shown in Figures 2.2 and 2.3 respectively.

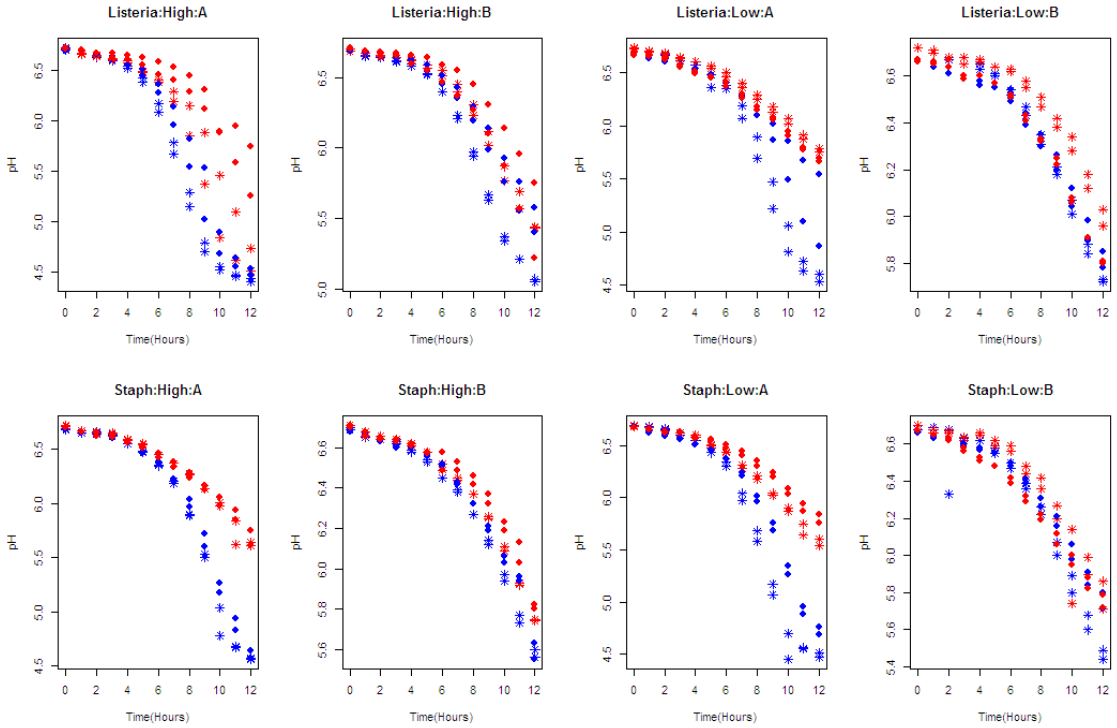

Figure 2.1 pH values

Legend: Raw milk – red, Pasteurised milk – blue; Trial 1 - ∗, Trial 2 - •

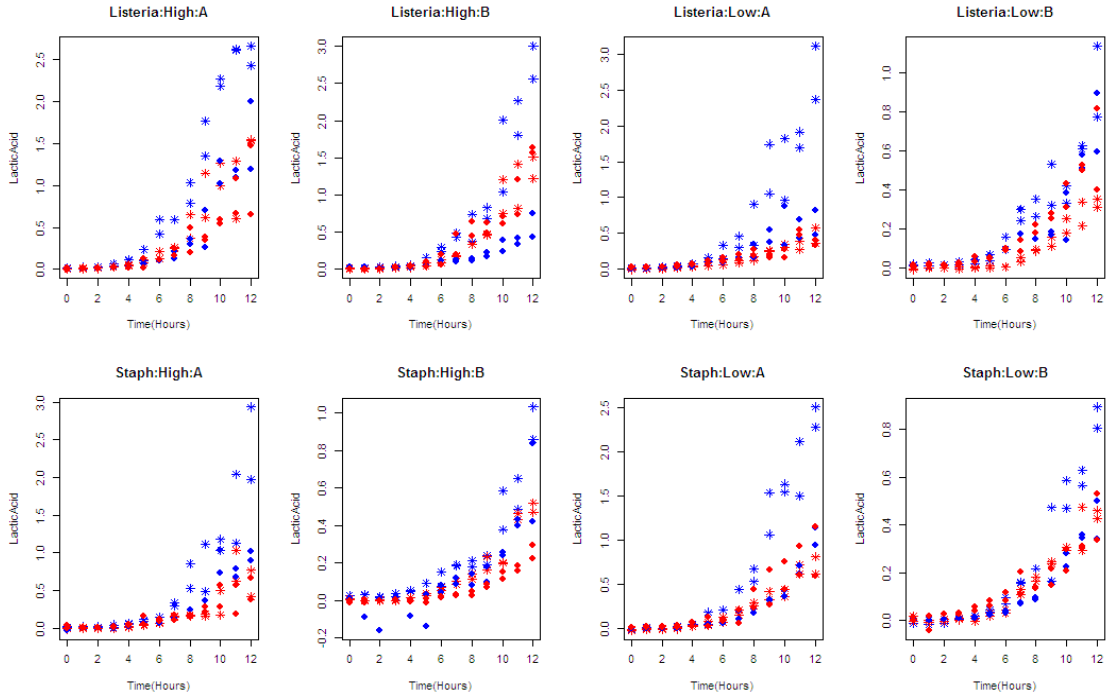

Figure 2.2 L-lactic acid

Legend: Raw milk – red, Pasteurised milk – blue; Trial 1 - ∗, Trial 2 - •

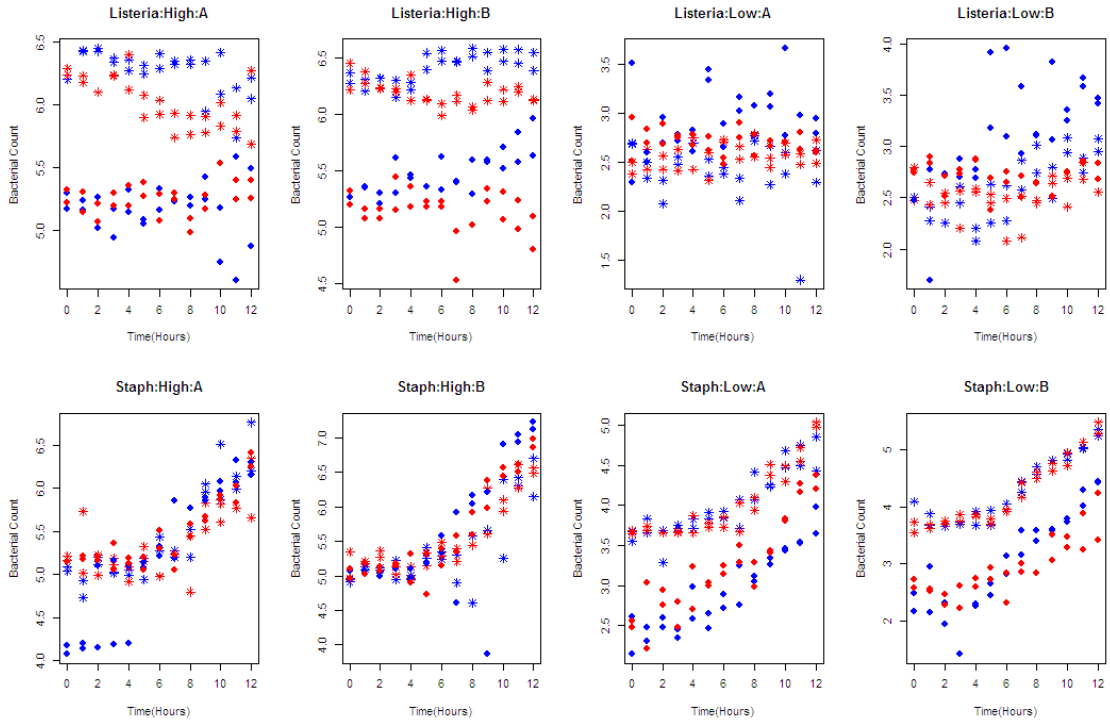

Figure 2.3 $\log_{10}$cfu/mL (bacterial count)

Legend: Raw– red, Pasteurised – blue; Trial 1 - ∗, Trial 2 - •

It is clear that, in general, challenge 1 results differ from change 2 results, in particular, at higher incubation hours for ph and lactic acid measurements. However, the difference in (log) bacterial count between the two trials is remarkable at all incubation hours except perhaps for a couple of scenarios.

This implies that duplicate samples from each of the two challenge trials could not be considered together as four independent replicates in the various ANOVAs. Hence, repeated measures AVOVAs with factors ‘Type of Milk’ and ‘Incubation time’ for each of pH, lactic acid and (log)bacterial count are shown in Tables 2.1, 2.2 and 2.3 respectively, separately for each of the 16 combinations of Challenge trials, Pathogen, Inoculum and Starter factors. Graphs of the comparisons used in the ANOVA analysis are shown in Figures 2.4, 2.5, and 2.6 respectively.

Table 2.1 Repeated measures ANOVA (pH values)

"Listeria:High:A:T1"

	numDF	denDF	F-value	p-value
Milk	1	2	11.235	0.0787
Time1	12	24	195.928	<.0001
Milk:Time1	12	24	6.970	<.0001

"p-values for Pasteurised vs Raw at 0 & 12"
1.0000 0.1672

“Listeria:High:B:T1"

	numDF	denDF	F-value	p-value
Milk	1	2	82.4	0.0119
Time1	12	24	1844.9	<.0001
Milk:Time1	12	24	57.6	<.0001

"p-values for Pasteurised vs Raw at 0 & 12"
1.0000 0.0000

“Listeria:Low:A:T1"

	numDF	denDF	F-value	p-value
Milk	1	2	123.46	0.008
Time1	12	24	409.27	<.0001
Milk:Time1	12	24	77.63	<.0001

"p-values for Pasteurised vs Raw at 0 & 12"
1.0000 0.0000

“Listeria:Low:B:T1"

	numDF	denDF	F-value	p-value
Milk	1	2	63.6	0.0154
Time1	12	24	916.2	<.0001
Milk:Time1	12	24	35.5	<.0001

"p-values for Pasteurised vs Raw at 0 & 12"
1.0000 0.0000

“Staph:High:A:T1"

	numDF	denDF	F-value	p-value
Milk	1	2	568.9	0.0018
Time1	12	24	592.6	<.0001
Milk:Time1	12	24	85.7	<.0001

"p-values for Pasteurised vs Raw at 0 & 12"
0.5394 0.0000

“Staph:High:B:T1"

	numDF	denDF	F-value	p-value
Milk	1	2	149	0.0066
Time1	12	24	3789	<.0001
Milk:Time1	12	24	31	<.0001

"p-values for Pasteurised vs Raw at 0 & 12"
0.2296 0.0000

“Staph:Low:A:T1"

	numDF	denDF	F-value	p-value
Milk	1	2	936.5	0.0011
Time1	12	24	749.1	<.0001
Milk:Time1	12	24	120.6	<.0001

"p-values for Pasteurised vs Raw at 0 & 12"
0.9156 0.0000

“Staph:Low:B:T1"

	numDF	denDF	F-value	p-value
Milk	1	2	6.21	0.1303
Time1	12	24	106.17	<.0001
Milk:Time1	12	24	2.32	0.0380

"p-values for Pasteurised vs Raw at 0 & 12"
0.9045 0.0007

“Listeria:High:A:T2"

	numDF	denDF	F-value	p-value
Milk	1	2	25.971	0.0364
Time1	12	24	141.477	<.0001
Milk:Time1	12	24	19.855	<.0001

"p-values for Pasteurised vs Raw at 0 & 12"
0.8521 0.0000

“Listeria:High:B:T2”

	numDF	denDF	F-value	p-value
Milk	1	2	0.404	0.5900
Time1	12	24	82.437	<.0001
Milk:Time1	12	24	0.418	0.9411

"p-values for Pasteurised vs Raw at 0 & 12"
0.9044 0.9680

“Listeria:Low:A:T2”

	numDF	denDF	F-value	p-value
Milk	1	2	2.10	0.2847
Time1	12	24	46.73	<.0001
Milk:Time1	12	24	1.89	0.0883

"p-values for Pasteurised vs Raw at 0 & 12"
1.0000 0.0018

“Listeria:Low:B:T2”

	numDF	denDF	F-value	p-value
Milk	1	2	0.0	0.8573
Time1	12	24	1130.2	<.0001
Milk:Time1	12	24	1.2	0.3436

"p-values for Pasteurised vs Raw at 0 & 12"
0.8439 0.6940

“Staph:High:A:T2”

	numDF	denDF	F-value	p-value
Milk	1	2	109.81	0.009
Time1	12	24	1156.05	<.0001
Milk:Time1	12	24	172.47	<.0001

"p-values for Pasteurised vs Raw at 0 & 12"
0.7195 0.0000

“Staph:High:B:T2”

	numDF	denDF	F-value	p-value
Milk	1	2	23.4	0.0402
Time1	12	24	748.7	<.0001
Milk:Time1	12	24	11.1	<.0001

"p-values for Pasteurised vs Raw at 0 & 12"
0.4527 0.0000

“Staph:Low:A:T2”

	numDF	denDF	F-value	p-value
Milk	1	2	242.8	0.0041
Time1	12	24	1589.3	<.0001
Milk:Time1	12	24	245.4	<.0001

"p-values for Pasteurised vs Raw at 0 & 12"
0.8751 0.0000

“Staph:Low:B:T2”

	numDF	denDF	F-value	p-value
Milk	1	2	3.8	0.1916
Time1	12	24	970.7	<.0001
Milk:Time1	12	24	4.2	0.0015

"p-values for Pasteurised vs Raw at 0 & 12"
0.8647 1.0000

Repeated measures ANOVA results in Table 2.1 show significant interaction effect ($p<0.05$) between type of milk and incubation time for all scenarios, except for Listeria:High:B:T2, Listeria:Low:A:T2 and Listeria:Low:B:T2. The significance of the interaction effect can be highlighted by the graphs shown in Figure 2.4. The difference in pH between raw and pasteurised milk become prominent at higher incubation times. Also, in the cases where the significance is high, raw milk produces higher pH values compared to pasteurised milk.

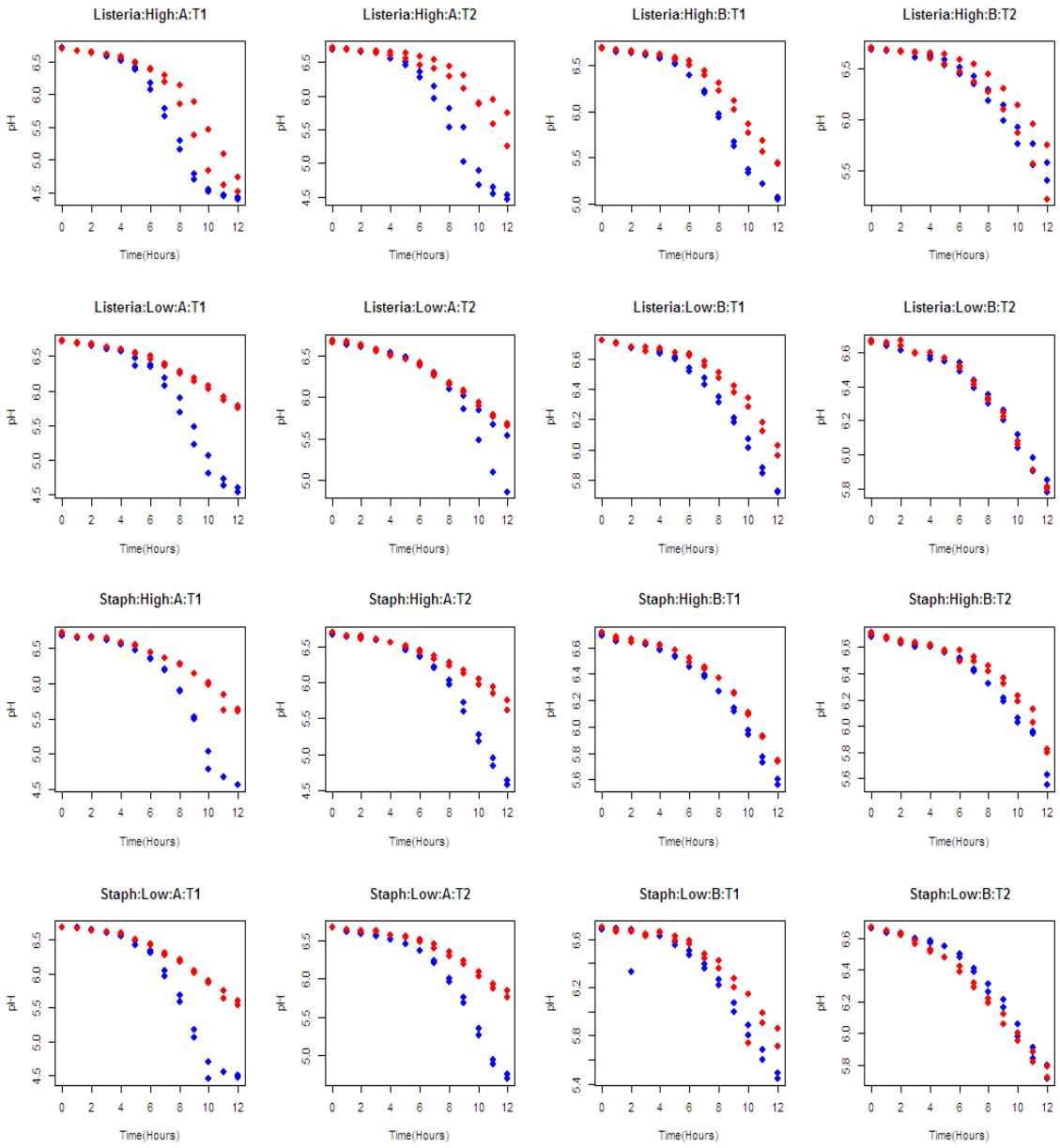

Figure 2.4 pH values with 16 scenarios

Legend: Raw milk – red, Pasteurised milk – blue

Table 2.1 also shows the p-values correspond to the significance of the difference between raw and pasteurised milk at 0 and 12 hours of incubation. In all 16 scenarios, the difference is not significant ($p>>>0.05$) at 0 hours of incubation. However, in most cases, the difference is significant ($p<0.05$) after 12 hours of incubation; the exceptions being the scenarios Listeria:High:A:T1, Listeria:High:B:T2, Listeria:Low:B:T2 and Staph:Low:B:T2. Note that, in scenario Listeria:High:A:T1, the interaction effect between type of milk and incubation time is significant, but no significant difference between raw and pasteurised milk is indicated at times 0 and 12 hours. This means that the difference in the two milk types at other times (intermediate incubation hours) may be significant.

Table 2.2: Repeated measures ANOVA (L-lactic acid values)

```
"Listeria:High:A:T1"
            numDF denDF  F-value p-value
Milk            1     2 104.2165  0.0095
Time1          12    24  95.5654  <.0001
Milk:Time1     12    24  11.4761  <.0001
"p-values for Pasteurised vs Raw at 0 & 12"
0.9441 0.0000

"Listeria:High:B:T1"
            numDF denDF  F-value p-value
Milk            1     2  29.5516  0.0322
Time1          12    24  45.1081  <.0001
Milk:Time1     12    24   4.3378  0.0011
"p-values for Pasteurised vs Raw at 0 & 12"
0.9589 0.0000

"Listeria:Low:A:T1"
            numDF denDF   F-value p-value
Milk            1     2  75.59441   0.013
Time1          12    24  24.34494  <.0001
Milk:Time1     12    24  12.07428  <.0001
"p-values for Pasteurised vs Raw at 0 & 12"
0.9496 0.0000

"Listeria:Low:B:T1"
            numDF denDF   F-value p-value
Milk            1     2  46.90235  0.0207
Time1          12    24  39.69292  <.0001
Milk:Time1     12    24   7.77873  <.0001
"p-values for Pasteurised vs Raw at 0 & 12"
0.7532 0.0000

"Staph:High:A:T1"
            numDF denDF  F-value p-value
Milk            1     2 12.64011  0.0708
Time1          12    24 22.82645  <.0001
Milk:Time1     12    24  6.57002  <.0001
"p-values for Pasteurised vs Raw at 0 & 12"
0.9217 0.0000

"Staph:High:B:T1"
            numDF denDF  F-value p-value
Milk            1     2  54.3595  0.0179
Time1          12    24  85.2402  <.0001
Milk:Time1     12    24   7.0701  <.0001
"p-values for Pasteurised vs Raw at 0 & 12"
0.6746 0.0000

"Staph:Low:A:T1"
            numDF denDF  F-value p-value
Milk            1     2 165.2289   0.006
Time1          12    24  78.0803  <.0001
Milk:Time1     12    24  22.5266  <.0001
"p-values for Pasteurised vs Raw at 0 & 12"
0.9987 0.0000

"Staph:Low:B:T1"
            numDF denDF  F-value p-value
Milk            1     2  25.6614  0.0368
Time1          12    24  62.2583  <.0001
Milk:Time1     12    24   5.2504  0.0003
"p-values for Pasteurised vs Raw at 0 & 12"
0.7458 0.0000
```

```
"Listeria:High:A:T2"
            numDF denDF  F-value p-value
Milk            1     2  1.40158  0.3581
Time1          12    24 26.41432  <.0001
Milk:Time1     12    24  1.53566  0.1791
"p-values for Pasteurised vs Raw at 0 & 12"
0.9786 0.0106

"Listeria:High:B:T2"
            numDF denDF  F-value p-value
Milk            1     2  65.8729  0.0148
Time1          12    24  42.5448  <.0001
Milk:Time1     12    24   9.5189  <.0001
"p-values for Pasteurised vs Raw at 0 & 12"
0.8415 0.0000

"Listeria:Low:A:T2"
            numDF denDF  F-value p-value
Milk            1     2  1.24751  0.3802
Time1          12    24 17.60753  <.0001
Milk:Time1     12    24  2.57203  0.0236
"p-values for Pasteurised vs Raw at 0 & 12"
0.9143 0.0142

"Listeria:Low:B:T2"
            numDF denDF   F-value p-value
Milk            1     2   0.03387  0.8709
Time1          12    24  25.44840  <.0001
Milk:Time1     12    24   0.66769  0.7644
"p-values for Pasteurised vs Raw at 0 & 12"
0.9756 0.1163

"Staph:High:A:T2"
            numDF denDF   F-value p-value
Milk            1     2  10.27220  0.0851
Time1          12    24  31.65047  <.0001
Milk:Time1     12    24   4.02881  0.0018
"p-values for Pasteurised vs Raw at 0 & 12"
0.7557 0.0001

"Staph:High:B:T2"
            numDF denDF  F-value p-value
Milk            1     2  9.23493  0.0934
Time1          12    24 16.04421  <.0001
Milk:Time1     12    24  3.05138  0.0096
"p-values for Pasteurised vs Raw at 0 & 12"
0.8909 0.0000

"Staph:Low:A:T2"
            numDF denDF  F-value p-value
Milk            1     2  0.26006  0.6608
Time1          12    24 41.36781  <.0001
Milk:Time1     12    24  0.93660  0.5289
"p-values for Pasteurised vs Raw at 0 & 12"
0.9056 0.1895

"Staph:Low:B:T2"
            numDF denDF  F-value p-value
Milk            1     2   3.5440  0.2005
Time1          12    24  41.1344  <.0001
Milk:Time1     12    24   0.6802  0.7536
"p-values for Pasteurised vs Raw at 0 & 12"
0.9739 0.7649
```

Repeated measures ANOVA results in Table 2.2 show significant interaction effect ($p<0.05$) between type of milk and incubation time for all scenarios for L-lactic acid production, except for Listeria:High:A:T2, Listeria:Low:B:T2, Staph:Low:A:T2, and Staph:Low:B:T2. The significance of the interaction effect can be seen in the graphs shown in Figure 2.5. In the cases where the significance is high, raw milk had low L-lactic levels compared to pasteurised milk except for Listeria:High:B:T2.

Table 2.2 also shows the *p*-values correspond to the significance of the difference between raw and pasteurised milk at 0 and 12 hours of incubation. In all 16 scenarios, the difference is not significant ($p>>>0.05$) at 0 hours of incubation. However, in most cases, the difference is significant ($p<0.05$) after 12 hours of incubation; the exceptions being the scenarios Listeria:Low:B:T2, Staph:Low:A:T2 and Staph:Low:B:T2.

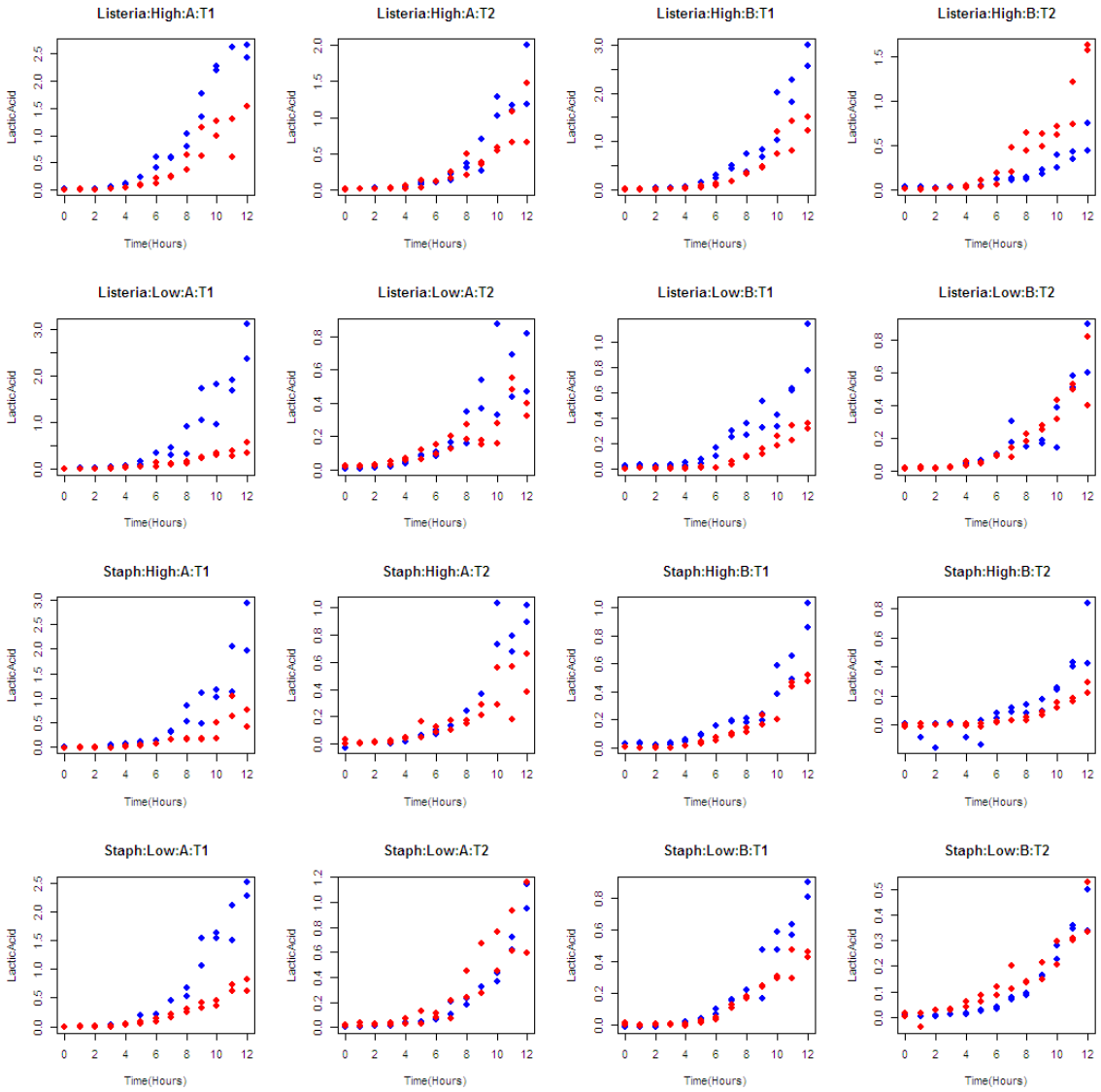

Figure 2.5 L-lactic acid values with 16 scenarios

Legend: Raw milk – red, Pasteurised milk – blue

Table 2.3 Repeated measures ANOVA (log_{10}bacterial counts)

"Listeria:High:A:T1"

	numDF	denDF	F-value	p-value
Milk	1	2	11.647	0.0762
Time1	12	24	3.993	0.0019
Milk:Time1	12	24	1.657	0.1413

"p-values for Pasteurised vs Raw at 0 & 12"
0.7809 0.3043

"Listeria:High:B:T1"

	numDF	denDF	F-value	p-value
Milk	1	2	36.53	0.0263
Time1	12	24	1.20	0.3350
Milk:Time1	12	24	6.85	<.0001

"p-values for Pasteurised vs Raw at 0 & 12"
0.8540 0.0003

"Listeria:Low:A:T1"

	numDF	denDF	F-value	p-value
Milk	1	2	21.479	0.0435
Time1	12	24	7.195	<.0001
Milk:Time1	12	24	6.449	0.0001

"p-values for Pasteurised vs Raw at 0 & 12"
0.0867 0.2990

"Listeria:Low:B:T1"

	numDF	denDF	F-value	p-value
Milk	1	2	3.454	0.2042
Time1	12	24	3.753	0.0028
Milk:Time1	12	24	3.016	0.0103

"p-values for Pasteurised vs Raw at 0 & 12"
0.3921 0.0104

"Staph:High:A:T1"

	numDF	denDF	F-value	p-value
Milk	1	2	2.615	0.2473
Time1	12	24	13.608	<.0001
Milk:Time1	12	24	1.740	0.1200

"p-values for Pasteurised vs Raw at 0 & 12"
0.5984 0.0452

"Staph:High:B:T1"

	numDF	denDF	F-value	p-value
Milk	1	2	3.463	0.2038
Time1	12	24	14.132	<.0001
Milk:Time1	12	24	0.217	0.9958

"p-values for Pasteurised vs Raw at 0 & 12"
0.4045 0.7101

"Staph:Low:A:T1"

	numDF	denDF	F-value	p-value
Milk	1	2	0.000	0.9924
Time1	12	24	43.982	<.0001
Milk:Time1	12	24	1.737	0.1206

"p-values for Pasteurised vs Raw at 0 & 12"
0.7434 0.0182

"Staph:Low:B:T1"

	numDF	denDF	F-value	p-value
Milk	1	2	0.43	0.5780
Time1	12	24	90.49	<.0001
Milk:Time1	12	24	0.55	0.8561

"p-values for Pasteurised vs Raw at 0 & 12"
0.1698 0.5020

"Listeria:High:A:T2"

	numDF	denDF	F-value	p-value
Milk	1	2	1.918	0.3003
Time1	12	24	0.261	0.9906
Milk:Time1	12	24	1.021	0.4610

"p-values for Pasteurised vs Raw at 0 & 12"
0.8628 0.4651

"Listeria:High:B:T2"

	numDF	denDF	F-value	p-value
Milk	1	2	45.04	0.0215
Time1	12	24	2.23	0.0459
Milk:Time1	12	24	3.02	0.0103

"p-values for Pasteurised vs Raw at 0 & 12"
0.9703 0.0000

"Listeria:Low:A:T2"

	numDF	denDF	F-value	p-value
Milk	1	2	5.899	0.1358
Time1	12	24	1.099	0.4042
Milk:Time1	12	24	1.111	0.3954

"p-values for Pasteurised vs Raw at 0 & 12"
0.4950 0.2897

"Listeria:Low:B:T2"

	numDF	denDF	F-value	p-value
Milk	1	2	12.9026	0.0695
Time1	12	24	2.5458	0.0248
Milk:Time1	12	24	3.8205	0.0025

"p-values for Pasteurised vs Raw at 0 & 12"
0.5846 0.0188

"Staph:High:A:T2"

	numDF	denDF	F-value	p-value
Milk	1	2	3.551	0.2002
Time1	12	24	19.471	<.0001
Milk:Time1	12	24	3.745	0.0029

"p-values for Pasteurised vs Raw at 0 & 12"
0.0006 0.6900

"Staph:High:B:T2"

	numDF	denDF	F-value	p-value
Milk	1	2	0.006	0.9460
Time1	12	24	15.600	<.0001
Milk:Time1	12	24	1.181	0.3496

"p-values for Pasteurised vs Raw at 0 & 12"
0.9036 0.5350

"Staph:Low:A:T2"

	numDF	denDF	F-value	p-value
Milk	1	2	30.399	0.0314
Time1	12	24	26.568	<.0001
Milk:Time1	12	24	0.724	0.7149

"p-values for Pasteurised vs Raw at 0 & 12"
0.5064 0.0284

"Staph:Low:B:T2"

	numDF	denDF	F-value	p-value
Milk	1	2	1.841	0.3077
Time1	12	24	22.266	<.0001
Milk:Time1	12	24	2.658	0.0200

"p-values for Pasteurised vs Raw at 0 & 12"
0.2379 0.0353

Repeated measures ANOVA results in Table 2.3 show significant interaction effect ($p<0.05$) between type of milk and incubation time for all scenarios for pathogen numbers, except for Listeria:High:A:T1, Listeria:High:A:T2, Listeria:Low:A:T2, Staph:High:A:T1, Staph:High:B:T1, Staph:High:B:T2, Staph:Low:A:T1, Staph:Low:A:T2, and Staph:Low:B:T1. The significance of the interaction effect can be observed in the graphs shown in Figure 2.6.

Table 2.3 also shows the *p*-values correspond to the significance of the difference between raw and pasteurised milk at 0 and 12 hours of incubation. In all 16 scenarios, the difference is not significant ($p>>>0.05$) at 0 hours of incubation except for Staph:High:A:T2. This observed difference is likely to be due to inoculum variation rather than any differences associated with the trial parameters. Significant differences ($p<0.05$) are observed at T=12 for scenarios Listeria:High:B:T1, Listeria:High:B:T2, Listeria:Low:B:T1, Listeria:Low:A:T2, Staph:High:A:T1, Staph:Low:A:T1, Staph:Low:A:T2, and Staph:Low:B:T2.

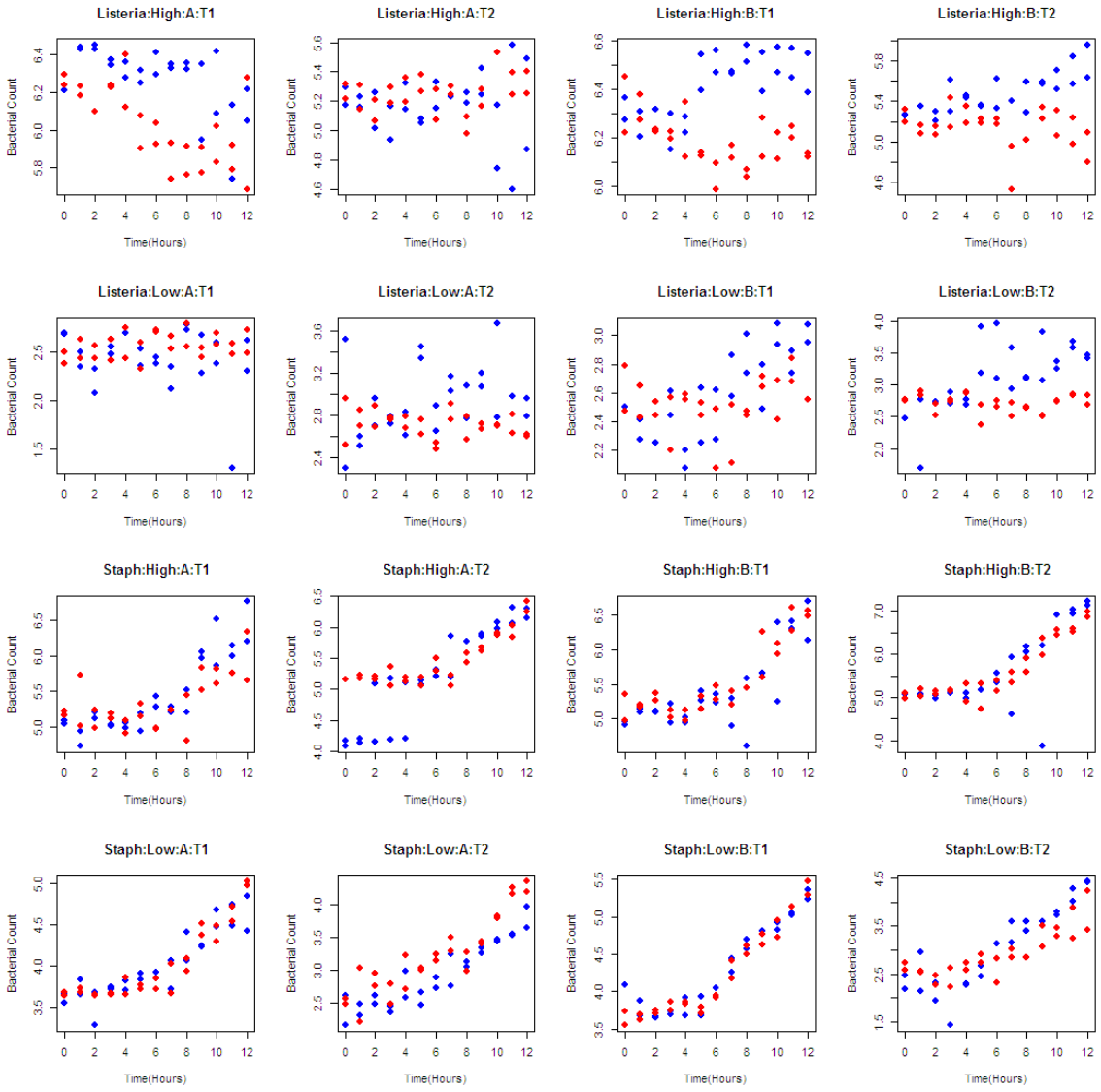

Figure 2.6: $\log_{10}$ cfu/mL (bacterial counts) with 16 scenarios

Legend: Raw milk – red, Pasteurised milk – blue