NON-PHAGE INHIBITION OF CHEESE STARTER LACTOCOCCI

A thesis submitted in total fulfillment of the requirements for the degree of Master of Applied Science (Dairy Technology)

by

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“...the exponential increase in our knowledge of what is in milk has led to little understanding of how things get there and why.”

Ephraim Y. Levine, 1995
Modern, large scale Cheddar cheese manufacture is dependent on reliable acid production by *Lactococcus lactis* subspecies *cremoris* and subspecies *lactis* starter cultures. Any inhibition of acid production may affect cheese quality, disrupt production schedules and reduce profitability.

The presence of antibiotic residues in manufacturing milk resulting from the treatment of mastitis in lactating cattle is a potential source of starter culture inhibition. Therefore, a range of antibiotic concentrations was assessed for measurable inhibitory effects on acid production and compared to the minimum detectable concentrations by approved screening test procedures. Antibiotics were selected from formulations approved for use on lactating cattle for the treatment of mastitis. Novobiocin, lincomycin, oleandomycin and oxytetracycline HCl, all non-β-lactam antibiotics, inhibited acid production of one or more *L. lactis* strains at antibiotic concentrations below the detectable limit of standard screening procedures. Depending on the antibiotic, either or both the *Bacillus stearothermophilus* (var. *calidolactis*) disk assay and/or the Delvo SP assay were ineffective at detecting the antibiotics at concentrations required to inhibit the starter strains. Consequently, antibiotic residues below the detectable limits of these testing procedures could cause significant starter culture inhibition, disrupting cheese making schedules.

Another potential source of starter culture inhibition is related to raw milk quality and the practice of refrigerated storage prior to processing. Previous studies differed as to whether the growth of psychrotrophic organisms would have a detrimental impact on subsequent acid production by starter bacteria employed in cheese manufacture. In this study, no inhibition of
acid production by a commercial *L. lactis* subsp. *cremoris* strain was evident when grown in milk that had undergone short term temperature abuse.

Antimicrobial systems native to bovine milk may also have an adverse impact on starter culture performance. The present study assessed the inhibitory effect of an activated lactoperoxidase system (LPS) on a range of *L. lactis* cultures. All of the strains were significantly inhibited when grown on reconstituted skim milk in the presence of an active LPS. Inhibition of acid production by strains grown on glucose was also observed, leading to further investigations to describe the inhibitory process.

A non-phosphoenolpyruvate phosphotransferase (PEP/PTS) dependent glucose transport system, first observed in 1980 in one *L. lactis* subsp. *lactis* strain, was hypothesised as a link in strain variations in LPS sensitivity. However, the LPS sensitive *L. lactis* subsp. *cremoris* strains tested did not take up glucose in a PEP depleted state, most likely due to their inability to utilise arginine as an ATP generating energy source. The questions remain unanswered whether *cremoris* strains possess this glucose transport mechanism and whether it could contribute to strain variations in LPS sensitivity. In a subsequent investigation, galactose phosphotransferase system (PTS) deficient *L. lactis* strain ATCC 7962 demonstrated log phase growth inhibition when grown on galactose in the presence of the model LPS. Previously reported LPS mediated effects on the glycolytic enzyme hexokinase do not appear to explain this result.

The present study confirmed strain variability in sensitivity to the model LPS among both *Lactococcus lactis* subspecies *lactis* and subspecies *cremoris* strains. Further, the observation that dithiothreitol significantly alleviated the inhibition of a highly sensitive *cremoris* strain, implicated the involvement of sulphhydryl groups as the target of the transient inhibitory factors. Data collected excluded the possibility that portions of the metabolic pathways involved in fructose and galactose metabolism are sensitive to the LPS in cells possessing

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*W. Packham*
PEP/PTS capability. This study also identified potential directions of further work to elucidate the mechanism(s) of LPS inhibition.
I certify that this thesis comprises only my original work, unless otherwise acknowledged and has not been submitted for any other academic credit.

signed: ---------------------------------------
Wayne Packham
I would like to express my gratitude to the people who have helped in many ways with this project. First of all, I thank Gaetan Limsowtin for paving the way for me to begin. Secondly, may I give credit for the assistance and encouragement I have received from my supervisors Malcolm Broome and Hubert Roginski in seeing to my success. In the darker moments, Ian Powell shed some light from his experience, insights and humour.

I would also like to note Hilton Deeth who assisted with the publication of my first article based on data generated from this study. Carlo Mason, a former colleague at ASCRC, contributed results from a DRDC funded project which were essential to building the impetus for this study. I also value the support given by my coworkers at the ASCRC and the staff at the Gilbert Chandler College of The University of Melbourne. May I also mention Matthew Knight and Dale Tomlinson of Victoria University of Technology for their assistance with the radioactivity lab work and Heather Craven of Food Science Australia for donated strains necessary for this project. Brett Mitchell of Murray-Goulburn Co-op. offered helpful information on raw milk quality. And to the cheese makers who shared with me their “real world” experiences, breathing life into my quest, I extend my thanks.

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<td>Australian Starter Culture Research Centre</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSDA</td>
<td><em>Bacillus stearothermophilus</em> disk assay</td>
</tr>
<tr>
<td>cfu</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>DFSV</td>
<td>Dairy Food Safety Victoria</td>
</tr>
<tr>
<td>DRDC</td>
<td>Dairy Research and Development Corporation</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>FAA</td>
<td>free amino acids</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acids</td>
</tr>
<tr>
<td>g</td>
<td>gravitational force</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>HTST</td>
<td>high temperature short time</td>
</tr>
<tr>
<td>IAA</td>
<td>iodoacetic acid</td>
</tr>
<tr>
<td>IDF</td>
<td>International Dairy Federation</td>
</tr>
<tr>
<td>LAB</td>
<td>lactic acid bacteria</td>
</tr>
<tr>
<td>LPS</td>
<td>lactoperoxidase system</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>MRL</td>
<td>maximum residue limit</td>
</tr>
<tr>
<td>OD</td>
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</tr>
<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
</tr>
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<td>parts per million</td>
</tr>
<tr>
<td>ppb</td>
<td>parts per billion</td>
</tr>
<tr>
<td>PTS</td>
<td>phosphotransferase system</td>
</tr>
<tr>
<td>PWM</td>
<td>pasteurized whole milk</td>
</tr>
<tr>
<td>RSM</td>
<td>reconstituted skim milk</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>subspecies</td>
<td>subspecies</td>
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1.0 General Introduction

1.1 Nutritional composition of milk

All species of mammals produce milk as an initial source of food for the neonate. Bovine (cow’s) milk is approximately 87% water. The remaining components are soluble carbohydrate, colloidally dispersed protein and emulsified fat, as well as dissolved salts, vitamins and trace elements. Lactose (4-O-β-D-galacto-pyranosyl-D-glucopyranose) is the principal carbohydrate in milk, forming approximately 4.6% of the total composition, or 40% of the total solids and is in true solution. Milk fat forms about 3.9% of the total composition by weight and is 98% triacylglycerides enveloped in a membrane formed from the secretory cells of the mammary tissue. This membrane facilitates the emulsification of the fat in the water phase. The principal protein of milk is casein, a complex structure composing approximately 2.6% of the total milk mass and just over 80% of the total milk proteins. The remaining proteins are usually referred to as whey proteins because they do not complex with casein at the curd formation of cheese making. Whey proteins include β-lactoglobulin, α-lactalbumin, serum albumin and immunoglobulins. Thus, the nutrient value of milk is appreciated by humans and therefore milk is widely used as a staple food source. Milk is also a useful medium for the growth of microorganisms capable of utilising its various components (Janness 1988).
1.2 Lactic acid bacteria

Lactic acid bacteria (LAB) play an essential role in the production of fermented milk products such as yoghurts and cheeses. In modern large scale manufacturing, LAB are primarily used to initiate the development of lactic acid and are therefore referred to as starter cultures. Other LAB strains are selected for their ability to produce various metabolic byproducts which contribute to the development of desirable flavours, textures and/or aromas in the final product. These organisms are often referred to as adjuncts. Non-starter lactic acid bacteria (NSLAB) can also grow to high numbers in cheese and while not deliberately added, are essential to the development of key product characteristics (Fox et al. 1998).

1.3 Starter cultures

1.3.1 Cheese manufacture

Starter cultures are used in cheese manufacture for the reliable production of lactic acid. In most cheese making contexts and for Cheddar cheese specifically, the development of lactic acid, in conjunction with the enzymatic action of rennet, is necessary for curd formation. Lactic acid production results in a decrease in pH which reduces the surface charge of the casein micelle, in turn resulting in a decrease in its solubility and the formation of a fragile network of hydrophobic bonds between the micelles. In addition, the reduced pH enhances rennet activity, which primarily hydrolyses $\kappa$-casein, also leading to gel formation. If the rate of acid production is correct, a gel will form encompassing the entire volume of the milk (Brulé et al. 2000).

Aside from the production of lactic acid, starter cultures can form flavour components such as acetic acid, acetaldehyde and diacetyl from the metabolism of carbohydrates, as well as contributing to the formation of other flavour compounds through their proteolytic activities. In addition, acid production by starter cultures helps to inhibit the growth of
undesirable organisms. Because of the important role starter cultures play in cheese manufacture, strain selection criteria include an association with favourable attributes in the end product or conversely, the omission of undesirable characteristics in the final product.

1.3.2 Starter types

Defined strain starter cultures may be composed of single or multiple strains which may have been characterised for key attributes such as rate of acid production, salt tolerance and proteolytic activities. Specific information on each strain is usually available from the starter supplier. Undefined cultures, where the precise composition of organism(s) in the culture is unknown, are also used although their use is usually restricted to the smaller manufacturers of European regional cheeses. In the larger industrial-scale plant however, starters generally consist of mixtures of defined strains.

1.3.3 Propagation

Traditionally, milk acidification in cheese or yoghurt making involved the use of a small amount of the previous day’s culture to inoculate the current day’s milk. For “cottage” scale production this form of starter culture propagation is cost efficient, but the day-to-day product variations are unacceptable at a larger scale, where greater control over each production process is required. In this case, starter cultures with desirable characteristics are maintained at the factory site and sub-cultured to progressively larger volumes when required. The final sub-culturing step is into a bulk starter vessel, which usually has external pH regulation and is of sufficient size to provide enough starter volume for the day’s production. This process allows the manufacturer to decide on strain rotation and mix composition. However, with each sub-culturing step there is a risk of contamination and in the case of mixed or undefined cultures, an increased likelihood of alterations in strain dominance. Concentrated cultures may be added directly to the bulk starter vessel as a single
sub-culturing step. These products are usually supplied deep frozen as single or mixed strain cultures and stored on site, offering the manufacturer a choice of strain on a daily basis. The cost of supplying cultures in this form is offset by the elimination of the repeated sub-culturing steps and the requirement of a starter laboratory on the manufacturing site. Both of these strategies require the starter culture to grow sufficiently in the sterilised medium (often steamed or UHT milk) used for starter propagation and the pasteurised milk in the cheese vat. For mixed or undefined cultures, growth in the two media may vary, altering the balance of strains within the mix. Highly concentrated (usually freeze dried) cultures can be added directly to the cheese vat. These products are more expensive but allow the use of mixed or undefined cultures, eliminate culture handling equipment altogether and reduce specialised training requirements. Manufacturers may choose this form of starter culture supply to avoid the capital outlay for propagation equipment (Limsowtin pers. comm., Canteri 2000).

1.4 **Microbiology of *Lactococcus lactis***

All genera of LAB are non-motile, non-spore forming, Gram positive, catalase negative and do not produce ATP aerobically. For the manufacture of Cheddar cheese, *Lactococcus lactis* strains are primarily used in starter cultures. The species is divided into two subspecies designated *lactis* and *cremoris*, the differentiation of which is routinely based on arginine metabolism. *Lactococcus lactis* subspecies *lactis* strains are able to produce NH$_3$, ornithine and citruline from arginine, whereas *Lactococcus lactis* subspecies *cremoris* possesses only two of the three arginine metabolising enzymes, as it lacks arginine deiminase (Crow and Thomas 1982). Taillez et al. (1998) used 16s rDNA sequencing to divide *Lactococcus lactis* into three groups and suggested that *cremoris* strains, all of which were in one group, most likely emerged through the loss of some specific characteristics. Nomura et al. (1999) added
that lactis, as opposed to cremoris strains, possessed glutamate decarboxylase which produces γ-aminobutyric acid from glutamate.

1.5 Lactose metabolism by *Lactococcus lactis*

*Lactococcus lactis* ferments lactose in a homofermentative fashion via the glycolytic pathway yielding L-lactate. ATP is produced strictly by substrate level phosphorylation, yielding 4 moles ATP and 4 moles lactate for each mole lactose metabolised. Lactose is primarily transported across the cell membrane via a phosphoenolpyruvate phosphotransferase system, is phosphorylated in transit and then cleaved intracellularly by phospho-β-galactosidase (pβgal). The glucose moiety is converted to triose phosphates through to pyruvate. The phosphorylated galactose moiety is converted in three steps to dihydroxyacetone-P and glyceraldehyde-3-P in the tagatose pathway. These triose phosphates are converted to pyruvate which in turn is reduced by lactate dehydrogenase to L-lactate via the oxidation of NADH to NAD⁺. This regeneration of NAD⁺ is essential for the dehydrogenation and phosphorylation of triose phosphates. In addition, four phosphoenolpyruvate molecules are generated for each lactose molecule transported, which in turn supplies the PEP/PTS (Monnet *et al*. 1996).

1.6 Inhibition of acid production

Starter culture inhibition results in reduced production of lactic acid by the strains present in the cheese vat or bulk starter vessel. Inhibition can result from native antimicrobial systems present in the milk, inadvertently added chemical residues, changes in milk composition or the presence of bacteriocins or bacteriophage (phage). It is critical in today’s cheese making situation that both milk producers and cheese manufacturers understand their respective processes to control or eliminate these potential inhibitory factors. Historically,
bacteriophage have been the most significant source of starter culture inhibition and have therefore been the subject of many studies.

1.6.1 Bacteriophage

Lactococcal bacteriophage can be divided into three groups on the basis of their morphology, i.e. head size and shape and tail length. Phage replication occurs in two distinct forms, usually described as the lytic (virulent) and lysogenic (temperate) cycles. The lytic cycle begins with the phage particle attaching to the sensitive host cell wall and injecting its DNA, which is then replicated and transcribed by the host organism leading to the production of new phage. Following host lysis and cell death, new phage particles are released.

The lysogenic cycle also begins with phage particle attachment and DNA injection, however, instead of the formation of new phage particles, the host cell incorporates the phage DNA into its own chromosome. Once in place, the cell becomes insensitive to infection by other phage particles and the organism will maintain a normal life cycle until the inert phage DNA is activated, the phage particles are assembled and the host cell is destroyed in the process (Fox et al. 2000). Host cell defenses to phage infection include inhibition of adsorption, modification of phage DNA and abortive infection. Cells that exhibit abortive infection resistance mechanisms typically die once infected and before new phage particles are released thereby limiting phage propagation (Allison and Klaenhammer 1998).

The development of acid in a phage positive culture is indistinguishable from that in a phage free culture from inoculation through to early log phase growth. Towards mid-to-late log phase of growth, as the infected cells begin to release new phage particles, acid production rapidly declines in comparison to that in the phage free culture. In the cheese vat, the presence of phage specific for the starter culture in use results in reduced acid production late in the cheese make. Subsequent fills of poorly sanitised vats lead to increasing phage titres and rapidly declining acid production. When phage infection of the bulk starter occurs,
the entire days’ production can be affected. However, these effects are well understood by large scale cheese makers and management. Improved factory design, strain selection techniques, asceptic starter propagation and other hygiene protocols, all regarded as strict necessities, have combined to reduce the import of bacteriophage infection (Powell 2002).

1.6.2 Non-phage inhibition

Bovine milk contains a number of antimicrobial systems designed to confer passive immunity to the calf and to provide protection to the interior of the mammary gland. Immunoglobulins are a significant source of passive immunity which is conferred to the calf and although a range of immunoglobulin types are secreted, of which IgG1 is the most significant by concentration, the inhibitory effects on starter cultures are eliminated if the milk is pasteurised. Lysozyme (EC 3.2.1.17) is a naturally occurring enzyme in milk that hydrolyses glycosidic bonds in Gram-positive bacterial cell walls. Again, as it is present in low concentration (in comparison to those in milk from other species) and is partially inactivated during pasteurisation, it is unlikely to have an adverse impact on starter activity in cheese manufacture. Lactoferrin binds iron, making it inaccessible to microorganisms but its contribution to any inhibition of starter cultures is unlikely since LAB do not possess haem-based enzymes. Other enzymes in milk bind vitamin B12, folate and riboflavin, but their significance as inhibitors of starter cultures is unknown. Of greater importance is lactoperoxidase (EC 1.11.1.7), an enzyme forming part of an antibacterial system which does survive pasteurisation and is known to be bactericidal (Jensen 1995).

Other sources of non-phage inhibition result from modern milk production, handling or storage practices. Dairy equipment cleaning and sanitising agents may inadvertently enter the milk if rinsing is ineffective. Other residues may result from non-veterinary treatments for fly or parasite infections where no withholding period for lactating cattle is required. Investigations into the likelihood of residues occurring from these practices and the
possibility of any such compounds inhibiting starter cultures has not been carried out to a
great extent.

Factors such as prolonged storage, elevated temperatures, shear forces and/or aeration
from stirring or pumping, may contribute to changes in milk components which in turn can
affect starter performance. Thus the potential for starter culture inhibition exists at all stages
of milk production.

1.6.3 Occurrence of non-phage inhibition

A number of studies have attempted to identify potential sources of inhibition in the
cheese vat. For example, Roginski et al. (1984a) conducted a survey of milks from three
collection areas in Victoria, Australia, noting a seasonal effect on starter performance. In a
further study by Roginski et al. (1984b), quaternary ammonium compounds used as dairy
sanitisers were assessed for their impact on starter cultures. In this case, significant inhibition
occurred only at higher concentrations. However, because the causes of non-phage inhibition
are still not clear, considerable scope remains for further investigation.

In a more recent study the ASCRC, in collaboration with the DRDC, conducted an
investigation into Cheddar cheese production at a large scale factory in northern Victoria
(Mason et al. 2001). Among the objectives of the study was the identification of starter
related difficulties not linked to the presence of bacteriophage. On-site logbooks and external
laboratory testing results relevant to Cheddar cheese production over two manufacturing
seasons (1997-1999) were reviewed. Starter culture inhibition was defined using any or all of
the following three parameters: (i) pH readings greater than 0.1 units above specifications,
(ii) extensions of greater than ten minutes on "cheese make" times to reach required
specifications and (iii) starter culture inoculum increases of >30 litres above normal doses as
compensation for slower previous vats.
During the 1997/1998 season there were a total of 43 days with inhibited production, 25 of these being attributed to non-phage problems, affecting some 441 vats (>9 million litres of milk). Data from the 1998/1999 production season showed that inhibition occurred on 21 production days, ten of which were non-phage in origin, affecting approximately 3.8 million litres of milk. These observations demonstrate that inhibition due to phage infection still occurs and indicate the extent and variability of non-phage inhibition encountered by Cheddar cheese production facilities of this size and technology.

1.7 Outline of project

The present study has been divided into three parts. In the first section the limitations of standard antibiotic detection systems when applied to milk for cheese making are examined while the second part explores the possibility of starter culture inhibition due to short term temperature abuse of milk prior to processing. Finally, the effect of an activated lactoperoxidase system on a range of *Lactococcus lactis* strains was investigated.
2.0 Materials and Methods

2.1 Chemicals and Equipment

Details of chemicals and reagents are listed in Appendix A9. Details of equipment model and manufacturer are listed in Appendix A10.

2.2 Media

2.2.1 M17 broth

M17 broth (Terzaghi and Sandine 1975) was prepared by mixing 37.25 g M17 powder in 1 litre de-ionized water, adjusting pH to 7.2 (using 1M NaOH or 1M HCl for pH adjustments of all media) and autoclaving at 121°C for 15 minutes. The required carbohydrate (lactose, fructose, glucose or galactose) was dissolved in deionised water at 40-100 times the required concentrations, filter sterilised and added to the broth to achieve a final concentration of 5g l⁻¹. The medium was stored at 4°C for no longer than eight weeks prior to use.

2.2.2 1/10 M17 broth

1/10 M17 broth (Terzaghi and Sandine 1975) was prepared by mixing 3.7 g M17 powder and 0.5g lactose in 1 litre de-ionized water, adjusting pH to 7.2 and autoclaving at 121°C for 15 minutes. The medium was stored at 4°C for no longer than eight weeks prior to use.

2.2.3 Nutrient agar

Nutrient agar was prepared by mixing 28g nutrient agar powder in 1 litre deionised water, adjusting to pH 7.4 and autoclaving at 121°C for 15 minutes. The molten agar was cooled to
55°C and slopes were poured in 35ml screw capped tubes. This medium was stored at 4°C for no longer than eight weeks prior to use.

2.2.4 Nutrient broth

Nutrient broth was prepared by mixing 13g nutrient broth powder in 1 litre de-ionized water, adjusting to pH 7.4 and autoclaving at 121°C for 15 minutes. This medium was stored at 4°C for no longer than eight weeks prior to use.

2.2.5 Pasteurized whole milk (PWM)

PWM (unhomogenised) was purchased from retail outlets as required and blended before use.

2.2.6 Plate Count agar

Plate count agar was prepared by mixing 17.5g plate count agar powder in 1 litre de-ionized water, adjusting to pH 7 and autoclaving at 121°C for 15 minutes. The medium was cooled to 55°C and approximately 20 ml poured into 85 mm petri dishes. The solidified plates were stored at 4°C for no longer than eight weeks prior to use.

2.2.7 Reconstituted Skim Milk (RSM)

RSM was prepared by mixing skim milk powder with de-ionized water to 10% w/v and autoclaving at 121°C for 15 minutes. The medium was stored at 4°C for no longer than eight weeks prior to use.

2.2.8 TYL broth

TYL broth, composed of 3%w/v tryptone, 1%w/v yeast extract, 0.5%w/v lactose, 0.2%w/v Lab Lemco powder, 0.5%w/v K2PO4 was prepared by blending with de-ionised water, adjusting to pH 6.5, filtering through Whatman no.1 filter paper and autoclaving at 121°C for 15 minutes. This medium was stored at 4°C for no longer than eight weeks prior to use.
2.2.9 YTD broth

YTD broth, composed of 2%w/v tryptone, 1%w/v yeast extract and 0.5%w/v glucose was prepared by blending in deionised water, adjusting to pH 7.0, autoclaving at 121°C for 15 minutes and stored at 4°C according to AS1766.3.11 (Standards Australia 1991).

2.3 Bacterial cultures

2.3.1 Bacillus stearothermophilus var. calidolactis

*Bacillus stearothermophilus* var. *calidolactis* was obtained from Dr. H. Craven, Food Science Australia, Werribee, Victoria, as colonies on a nutrient agar slope. This organism was stored on a nutrient agar slope at 4°C and maintained in YTD broth according to AS1766.3.11 (Standards Australia 1991).

2.3.2 Lactococcus lactis strains

*Lactococcus lactis* strains (Appendix A1) used in Australian large scale Cheddar cheese manufacture were obtained from the Australian Starter Culture Research Centre culture collection. *Lactococcus lactis* subsp. *lactis* strain ATCC 7962 (LMG 8525) was received from the Belgian Laboratorium voor Microbiologie, Universiteit Gent. Starter organisms were stored at -40°C in RSM as diluted cultures. When required, each culture was incubated at 25-30°C and subsequently maintained at 25-30°C by sub-culturing in RSM daily.

2.3.3 Pseudomonas strains

*Pseudomonas fluorescens* strain 113 and *Pseudomonas fragi* strain 71 were obtained on nutrient agar slopes from Dr. H. Craven, Food Science Australia, Werribee, Victoria and were stored at 4°C. Colonies were picked into nutrient broth and grown at 25°C for 24 hours and sub-cultured daily for at least two days prior to use.
2.4 Determination of \textit{L. lactis} activity

A 2\% (v/v) inoculum of \textit{Lactococcus lactis} cultures grown overnight in RSM was added to 10 ml (final vol.) of RSM. The cultures were incubated in 16mm (inside diameter) glass test tubes at 32\degree C in a waterbath for 5 hours to ensure that the cultures had reached log phase of growth (appendix A2 and A3). Endpoint pH was measured using a calibrated electrode and mean values were determined from experiments performed in triplicate. A pH increase of \( \geq 0.1 \) relative to the respective control was taken as an indication of inhibition (Roginski \textit{et al.} 1984a).

Continuous pH monitoring was carried out using calibrated double junction pH electrodes connected to a powered pH signal converter and a data acquisition system.

2.5 Antibiotics

The antibiotics ampicillin, cloxacillin, cefuroxime, penicillin G, lincomycin, oxytetracycline HCl, novobiocin, neomycin and oleandomycin were selected from the list of approved formulations for lactating cattle (National Registration Authority 1998, Appendix A12 and A13). All antibiotics were prepared by dilution in de-ionized water such that the addition of a 100 \( \mu l \) aliquot to a 10 ml final volume gave the target concentration. An equivalent aliquot of de-ionized water served as a control.

2.6 Antibiotic Detection Systems

2.6.1 Delvo SP

The Delvo SP kit was obtained and the assay carried out in duplicate according to the manufacturer’s specifications. The test sample (1.0 ml) was placed onto the surface of an agar plug impregnated with \textit{B. stearothermophilus} spores and containing a pH indicator. After incubation at 64\degree C for approximately 2.5h (maximum time determined by negative
control), a colour change in the agar indicates acid production from growth of the bacillus while no colour change indicates inhibition of acid production.

2.6.2 BSDA

The *B. stearothermophilus* disk assay (BSDA) was carried out according to AS1766.3.11 (Standards Australia 1991). A paper disk saturated in the test sample was placed on an agar plate into which an aliquot (1ml) of fresh culture of the test organism had been seeded. Following incubation at 55°C for a minimum of 2.5h the plates were inspected for zones of inhibition surrounding the disks. Quantitative measurements may be made against a standard curve comparing zone diameter and antibiotic concentration.

2.7 Short term temperature abuse of PWM

Three litres of pasteurised whole milk (PWM) were blended and aliquoted to 1l volumes. One ml of an overnight culture of *Pseudomonas fluorescens* and *Pseudomonas fragi* was inoculated separately into 1l of PWM milk. The uninoculated litre was used as the control medium. These milks were stored at 70°C for six days. Daily sub-samples were removed for analysis commencing immediately after inoculation (day zero).

2.7.1 Cell counts

Cell counts of both *Pseudomonas* spp. were performed by diluting the samples from each of the inoculated milks in 1/10 M17 broth. Sample aliquots (1 ml) from the serial dilutions were placed in 85mm Petri dishes. Subsequently, approximately 20 ml of melted plate count agar, cooled to 50°C, was poured into the plates. The plates were mixed gently, allowed to set and incubated at 70°C for up to 7 days. Colonies were counted on plates with between 30 and 300 colonies. This procedure was performed in triplicate for each dilution tested.
2.7.2 Protein degradation products

The extent of protein degradation in milk samples was estimated utilizing trinitrobenzene sulfonic acid (Samples et al. 1984). The cultured milk samples (10ml) were clarified by mixing with 24% trichloroacetic acid (5ml), letting stand for 30 minutes and filtering through Whatman number 1 filter paper. Filtrate (0.5 ml) was mixed with 0.5 ml 5% sodium citrate, 1.65 ml 0.12M Na Borate solution (in 0.12M NaOH), 0.25mL trinitrobenzene sulfonic acid, 0.5 ml 2M HCl and 0.5 ml 0.001M Na sulfite solution (in 0.1M NaH₂PO₄).

Spectrophotometric measurements of the resulting solutions were made at 420 nm. This procedure was performed in triplicate for each cultured milk sample. Measurements were compared to a standard curve from a series of known glycine concentrations.

2.7.3 Free fatty acids

Free fatty acid (FFA) assays of each milk sample were performed according to Deeth et al. (1975). A sample (6.0ml) of the milk, previously warmed to 30°C, was mixed with 8.0 ml water, 12.0 ml petroleum ether and 20.0 ml of “extraction mixture” (40 parts isopropanol, 10 parts petroleum ether and one part 4N H₂SO₄). The mixture was vigorously shaken for 15 seconds and allowed to separate. The top layer volume was measured and a sample titrated with 0.02N methanolic KOH. This procedure was performed in triplicate for each cultured milk sample.

2.7.4 L. lactis “activity” assay in pre-cultured PWM

Each cultured milk sample was batch pasteurised at 63°C for 30 minutes. Activity assays were performed in the pre-cultured milks as described previously (section 2.4) using Lactococcus lactis subsp. cremoris strain ASCC 47.
2.8 Sensitivity to model LPS

A selection of starter strains were assessed for their response to a model LPS by comparing activity (section 2.4) in RSM with added LPS. Strains exhibiting maximum difference in sensitivity to the model LPS system (compared to their respective controls) were selected for further study.

2.8.1 Components of the model LPS

LPS component concentrations were: 65 nM lactoperoxidase, 100 μM thiocyanate and 100 μM H₂O₂ (Roginski et al. 1984a). Each component was diluted in de-ionised water so that a 100μl aliquot into 10ml (final volume) gave the desired concentration.

2.8.2 L-lactate determination

Starters selected for their varying response to the model LPS were grown in M17 broth with added lactose, glucose, fructose or galactose at 5g l⁻¹. Typical growth curves of two of the selected *L. lactis* strains in the M17 broths are shown in appendices A4-A8 using change in optical density as the measure of growth. Overnight cultures were diluted to 2% and incubated at 32°C for 5 hours +/- LPS before determination of L-lactate production. Where necessary, samples were diluted in de-ionized water and cells removed using a 0.45 μm syringe filter. L-lactate was measured spectrophotometrically using a 96 well microplate reader at 340 nm using a D and L- lactate enzymatic assay kit. Analysis was carried out on triplicate experiments (including culture negative controls) and was compared to a standard curve as determined by the plate reader operating software.

2.8.3 Optical density measurements

Cultures maintained in RSM (as described in section 2.3.2) were sub-cultured into M17 broth + fermentable sugar (5g l⁻¹) and incubated overnight at 30°C prior to the assay. The overnight cultures were then diluted with M17 broth + fermentable sugar (5g l⁻¹) to give a
final concentration of 2% and incubated at 32°C for up to 10 hours. Samples (3.0ml) were taken at regular intervals and the OD determined spectrophotometrically at 600 nm against deionised water. Any sample at or above an OD reading of 0.9 was diluted in deionised water and an OD value was calculated from the reading using the dilution factor.

2.8.4 Cell free extract preparation

Strains were grown to late log phase in M17 broth with lactose, harvested by centrifugation and washed twice with ice cold 0.05M phosphate buffer (pH 7.0) before re-suspension at 1/10th the original volume in the same buffer. Cell free extracts were prepared by adding to the washed cells 0.1mm glass beads (approximately 4:1 cells to beads by volume to weight) and sonicating at maximum tuned energy for 5 minutes using 30 second pulses (30 seconds on 30 seconds off) with the cell solution suspended in ice water throughout sonication. An aliquot of sonicated suspension (1ml) was clarified by centrifugation for 5 minutes at 13,000 g at room temperature after which an aliquot of the supernatant was pipetted into a clean vial and placed in ice water.

2.8.5 NADH peroxidase assay

NADH peroxidase was determined according to Anders et al. (1970). To an anaerobic cuvette was added: 1.25ml 0.05M sodium phosphate-citrate buffer (pH 5.5), 100μl 3mM NADH, 400μl cell free extract (prepared as per 2.8.4) and 650μl deionised water. The reaction mixture was flushed gently with nitrogen using a needle fitting and 100μl of 30mM H₂O₂ was added to start the reaction. The reaction was followed spectrophotometrically at 340nm and continuously plotted using a chart recorder set on 0.5 volts full deflection.

2.8.6 Protein determination

The total protein concentration of cell free extracts (prepared as per 2.8.4) was determined according to Bradford (1976). The protein dye reagent was reconstituted in
deionised water and filtered through Whatman 1 paper. Samples of cell free extract (100\(\mu\)l) were mixed with the reagent (5.0ml) and held at room temperature for 30 minutes before measuring OD at 595nm. The results were compared to a standard curve using bovine serum albumin prepared in deionised water.

2.8.7 Glucose uptake by PEP depleted culture

Non-PEP/PTS mediated glucose uptake was measured according to Thompson (1980). Cells were grown to mid-log phase in 200ml M17 + galactose broth (5g l\(^{-1}\)) and harvested by centrifugation for 5 minutes at 10,000 g at 4\(^\circ\)C. Cells were washed twice in the same volume of 0.01M MgSO\(_4\) at ice water temperature and the pellet was resuspended in approximately 3ml of 0.01M MgSO\(_4\) to obtain a thick cell suspension. The cells were then diluted to approximately 200\(\mu\)g dry weight ml\(^{-1}\) as determined by optical density (Appendix A9) in 0.1M Trizma®-maleate buffer (pH 7). Iodoacetate was added to this suspension to give a final concentration of 10mM and the solution was held at room temperature for 10 minutes. The suspension was divided equally into two volumes, with one half held in ice water while to the other half was added 2-deoxy-D-glucose to a final concentration of 2mM which was held for a further 10 minutes at room temperature before it was chilled in ice water. Both solutions were centrifuged as described previously, the supernatant removed and the cell pellets resuspended in 0.1M Trizma®-maleate buffer (pH 7). Iodoacetate and arginine were added to give a final concentration of 10mM and 2mM respectively. Iodoacetate, 2-deoxy-D-glucose and arginine stock solutions had been prepared such that the addition of 100\(\mu\)l to 10ml final volume gave the desired concentration.

This reaction mixture was warmed to 32\(^\circ\)C and 40\(\mu\)l D-[\(U\)-\(^{14}\)C]glucose and 100\(\mu\)l glucose solutions were added to achieve a final glucose concentration of approximately 0.2mM. Samples (1.0ml) were withdrawn at intervals, filtered through 0.45\(\mu\)m nitrocellulose filter disks and rinsed with 4ml of 0.01M MgSO\(_4\). The disks were then placed in a
scintillation vial to which biodegradable scintillation fluid (3.0ml) was added before placing the vials in a scintillation counter for 1 minute each. Calculations of glucose uptake were based on 1Ci equalling $2.2 \times 10^{12}$ disintegrations per minute (Brady and Holum 1993). The molar ratio of $^{14}$C glucose to glucose was 0.0134:1. The conversion ratio of volume protoplasm to cell dry weight was 1.67:1 (ml:mg) according to (Thompson 1976). *L. lactis* subsp. *lactis* strain ASCC 249 (ML3) was used as control.

### 2.9 Statistical analysis

All statistical results given were calculated from experiments performed in triplicate. When presented graphically, calculated means are given as horizontal bars. Two standard deviations (+/-) were calculated and when presented graphically are given as vertical bars. Probability analyses (student t-test) and $R^2$ values were calculated using spreadsheet software.

### 2.10 Temperature monitoring

Temperature monitoring was performed by placing a type K thermocouple probe into appropriate pilot tubes. The probe was connected to a calibrated digital electronic thermometer.
“To date there has been no published evidence that the economic benefits of antibiotic treatment of mild clinical mastitis outweigh the risks and costs.”

Walter Guterbock, 1994

3.0 Limitations of standard antibiotic detection systems

3.1 Mastitis

Mastitis, or the inflammation of mammary tissue, is the most prevalent bovine disease in the dairy industry, occurring on a worldwide scale as shown by data from several dairy regions. For example, a survey of dairy farms in Ontario, Canada revealed that overall, 19.8% of cows experienced one or more cases of clinical mastitis (requiring intervention) during lactation (Sargeant et al. 1998). Similarly, a U.S. study showed 29% of heifers exhibited clinical mastitis at breeding age while 97% of breeding age heifers and pregnant heifers demonstrated intramammary infections in 75% of quarters (Nickerson et al. 1995). Thus a significant proportion of cattle are already infected prior to entering productive stages of the life cycle. Further evidence of this was shown in one Swedish study where 15% of cattle at the beginning of their productive phase exhibited clinical mastitis (Oltenacu and Ekesbo 1994). Elsewhere, Jordanian cattlemen can expect to treat 3.3 cases of clinical mastitis per 100 cows per month for herds over 59 cows (Lafi et al. 1994) while a Finnish study showed that more severe mastitic conditions can occur in 17% of lactations and recurrence of the disease can be expected in 14% of animals (Rajala and Grohn 1998). Year to year variations in overall incidence can also occur, however. Another Finish study reported
a 10% reduction in the incidence of mastitis in surveys conducted in 1988 and 1995, indicating a possible positive trend for the control of the disease (Myllys et al. 1998). Large percentages of cattle appear predisposed towards mastitis as Mycoplasma bovis, an organism believed to predispose the udder to disease caused by mastitis pathogens, was found in 43 and 62% of herds in Victoria and Queensland respectively (Ghadersohi et al. 1999).

### 3.2 Antibiotics in the dairy industry

The prevention or treatment of mastitis in lactating cattle has been the principal reason for the use of antibiotics in the dairy industry since the 1940s (Hady et al. 1993). However, the success of this strategy is debatable. A clinical or bacterial cure may be defined as elimination of symptoms or a significant reduction in pathogen population. Wilson et al. (1999) found only a 10% advantage when comparing antibiotic treated cases to spontaneous bacterial cure rates when streptococcal species were the causative organisms. Guterbock et al. (1993) compared antibiotic intervention and oxytocin therapy (a hormone used to enhance milking out), finding no significant difference between the cattle groups. Owens et al. (1997) confirmed the low cure rate for Staphylococcus aureus intramammary infections, an organism present in 15.4% of heifer (pre-productive females) quarters (Owens et al. 2001). Collectively, these results indicate the difficulties in achieving a successful mastitis treatment (Malmo 1992).

### 3.3 Costs to producers

Reductions in output and overall milk quality due to mastitis make the condition the most costly disease to the producer (Wells et al. 1998). Australian dairy farmers, it is estimated, loose more than A$130 million annually to poor udder health, with mastitis being the major contributing factor (Brightling et al. 1998). In the U.S., annual losses to producers from sub-clinical mastitis alone are estimated at U.S.$1 billion (Ott 1999). U.S. and Australian
authorities insist that dairy herd health is the most important issue in milk production, impacting product quality and ultimately the viability of their respective industries (Chambers 1998, Brightling et al. 1998).

### 3.4 Antibiotic residues in milk

One of the major drawbacks associated with antibiotic intervention for the treatment of mastitis, has been the possibility of residues entering the food chain. Fabre et al. (1995) reported that the use of antibiotics for the treatment or prevention of mastitis accounted for 88% of inhibitor residues in milk while Rüegg and Tabone (2000) illustrated a relationship between high somatic cell count milk (an early indicator of mastitic conditions) and antibiotic residue violations. However, the extent of this problem is still unclear. The American Veterinary Association reported that in 1996, of 3.3 million tankers tested, only 3,507 had violative antibiotic levels, leaving 99.9% of all tankers clear of residues (Smith 1997). Other workers argue that monthly on-farm testing is too infrequent to provide an accurate report of residue occurrences (Mellenberger 1997).

Much higher contamination rates were shown in one U.S. survey of retail milk utilising highly sensitive procedures which reported that 63% of the samples contained one or more residues (Brady and Katz 1988). In another survey, involving both the U.S. and Canada, 86% of milk samples delivered a positive result for one or more antibiotics (Collins-Thompson et al. 1988).

Determining the cause(s) of antibiotic residues in milk is a prime concern for the dairy industry. In one study conducted in Ontario, Canada farms which had tested positive for residues were compared to farms that had consistently produced negative results over the test period. A positive correlation was demonstrated for residue-positive farms with the employment of part-time employees (relief milkers) and the estimation of the frequency of antibiotic use. In contrast, residue-negative farms more consistently used separate milking
equipment on treated animals, rather than relying on diverting milk from the bulk tank, when compared to residue-positive farms (McEwan et al. 1991).

A mandatory post-treatment withholding period is used to prevent residues entering milk for human consumption. A standard number of milkings are discarded to ensure the treatment compounds are eliminated. However, a New Zealand survey of milks from first milkings post withholding period reported that 11.5% of samples produced positive results using the Delvo SP antibiotic test kit, with 76.5% of these being confirmed residue positive. In addition, 4.9% of first milking samples after drying off therapy tested positive using the Delvo SP antibiotic test kit, with 80% of these being confirmed positive (Ferguson 2001).

In the case of antibiotic contamination in food products such as milk, most guidelines are drawn up from a human health perspective, with maximum residue limits often set by the detection limits of standard assays (National Registration Authority 2001). However, the European Agency for the Evaluation of Medicinal Products has recently introduced residue determination guidelines which reflect the sensitivity of live bacterial cultures used in the dairy processing environment (EMEA 2001).

3.5 Impact on starter cultures

Antibiotic residues are known to have an inhibitory effect on acid production by starter lactic acid bacteria in the processing environment (Marth and Ellickson 1959). For example, penicillin residues at 0.1 and 0.05 units ml\(^{-1}\) have been found to cause a deterioration in cheese quality and delayed acid production during cheese manufacture respectively (Whitehead and Lane 1956). Poor acid development in the cheese vat leads to higher moisture retention in the curd and may lead to subsequent growth of pathogenic organisms during maturation (Bergère and Lenoir 2000). The extent to which LAB are inhibited by antibiotics was demonstrated by Katla et al. (2001). When 189 LAB isolates used in the dairy industry, from the genera Lactococcus, Streptococcus, Leuconostoc and Lactobacillus, were
tested for sensitivity to 14 antibiotics, only one strain (a *Lactobacillus*) was found to be resistant to one antibiotic (streptomycin). Lactic acid bacteria employed in the fermented meat industry have also been shown to be susceptible to antibiotic residues (Holley and Blaszyk 1998).

### 3.6 Standard antibiotic detection systems

Procedures for antibiotic detection have been developed and introduced into the dairy industry in order to identify milks containing antibiotic residues (Mitchell *et al.* 1998, Bishop and White 1984). Many antibiotic screening assays are based on the growth of sensitive organisms. Typically, the test organism produces acid as a metabolic byproduct which causes a colour change in the growth medium. In the presence of an inhibitory substance, acid production by the test organism is delayed and the anticipated colour change does not occur within either a defined time period or when compared to a negative control.

A variety of sensitive organisms have been proposed as indicators. Geleta *et al.* (1984) compared *Sarcina lutea* and *Bacillus subtilis* to *Bacillus stearothermophilus* for penicillin sensitivity, finding the later more sensitive overall. It was also found that penicillin g was detected in some samples of post-treatment milk one to three milkings beyond the discard time.

In Australia, the *Bacillus stearothermophilus* var. *calidolactis* disk assay (BSDA) has been adopted as the standard method for antibiotic detection (Standards Australia 1991). Other procedures based on *B. stearothermophilus*, such as the Delvo SP, have also been developed (Gilbertson *et al.* 1995; Muller and Jones 1993). The convenience of the Delvo SP has led to its widespread use in the dairy industry. According to an ASCRC survey of six major milk processors in south eastern Australia (NSW, Vic. and Tasmania), all reported using the Delvo SP assay for tanker testing (Mason, unpublished).
3.7 Advantages of Bacillus stearothermophilus detection systems

*B. stearothermophilus* is highly sensitive to β-lactam antibiotics, being inhibited by ampicillin at concentrations around 5 ppb (Heeschen *et al.* 1991). Although mastitis treatments are often combinations of more than one type of antibiotic, the assay has demonstrated no reduction in sensitivity due to a number of antibiotic combinations (Luitz *et al.* 1996). Other workers have shown the versatility of these systems, validating the BSDA for caprine milk (Zeng *et al.* 1998) and the Delvo SP, with modifications, for liquid whey (Diserens *et al.* 2002). The Delvo SP also reduces the likelihood of false positives caused by natural inhibitors (such as lysozyme and lactoferrin) by shielding the test organism from all but unusually high concentrations of these inhibitors which occur in colostrum or mastitic milk (Beukers 1993).

3.8 Disadvantages of Bacillus stearothermophilus detection systems

The sensitivity of the BSDA towards other antibiotic groups varies widely. For example, the BSDA detects the aminoglycoside dihydrostreptomycin at concentrations of 13 ppm and is completely insensitive to some antibiotics (Heeschen *et al.* 1991). Similarly, the Delvo SP assay can detect β-lactams at concentrations up to 1000 times lower than for other antibiotics such as dihydrostreptomycin (Honkanen-Buzalski and Reybroeck 1997). Hozová and Greifová (1995) found that *B. stearothermophilus* is insufficiently sensitive to tetracyclines. McGrane *et al.* (1996) reported that the Delvo SP assay had less than a 50% chance of detecting tetracyclines at the European Union MRL and was completely insensitive to chloramphenicol. Therefore, detection systems based on single organisms may be insufficient for broad spectrum detection. Also, confounding factors may lead to false positive results, especially when applied to milk from individual animals. For example, Van Eenennaam *et al.* (1993) confirmed this likelihood when applied to high somatic cell count milk.
3.9 Alternative antibiotic detection systems

Alternatives to the BSDA and Delvo SP assay include the T101 Streptococcus thermophilus-based antibiotic detection system (Valiotest). This assay is reported as being sensitive to β-lactams, tetracyclines and dihydrostreptomycin but requires up to twice the incubation period (4-5 hours) of the Delvo SP assay and the BSDA (Heeschen et al. 1991). Highly sensitive microbiological systems based on multiple organisms have also been assessed for milk screening. Nouws et al. (1999) investigated a seven plate system against 48 antibiotics, listing those not well detected, although none of these are approved for use in Australia. In an other study, Nouws et al. (1998) determined that the Charm HVS, a receptor assay, was unsuitable for tetracycline detection at concentrations below 150ng ml⁻¹, and that the B. cereus test plate was less expensive and more reliable for specific tetracycline detection.

Electrochemiluminescent, chromatographic and immunosorbent assays that are highly sensitive to multiple antibiotic groups have also been developed (Liang et al. 1996; Schenck and Calery 1998; Carlsson 1993; Charm and Ruth 1993) although there may be technical and/or cost disadvantages if they are used for routine screening (Suhren 1995).

3.10 Antibiotics in mastitis formulations

Non-β-lactam antibiotics make up a significant proportion of lactating cattle mastitis preparations used worldwide (McGrane et al. 1996). According to a survey conducted by the ASCRC, 164 dairy farms supplying a major milk processor in northern Victoria reported that non-β-lactam antibiotics made up approximately 64% (by frequency of use) of the therapeutic components of the intramammary treatments administered (Mason et al. 2001). Similarly, a 1999 survey to determine antibiotic dispensations of Australian veterinary practices showed that nationally, non-β-lactam antibiotics comprised >70% of mastitis
treatments for lactating cattle (Long 2001). In 1998, four out of seven formulations available for lactating cow therapy contained at least one non-\(\beta\)-lactam component (National Registration Authority 1998, appendix A12).

### 3.11 Aim of present study

In Australia, large-scale Cheddar manufacturers implement antibiotic detection systems to identify milk samples with contaminating antibiotic residues. The aim of this section was to determine whether commonly used antibiotic detection systems could detect the complete range of antibiotics approved for use on lactating cattle at concentrations below those which could adversely affect starter culture activity.

### 3.12 Results

According to the published and experimental antibiotic detection data, the Delvo SP is more sensitive than the BSDA for the detection of most antibiotics (Table 3.1). It is also interesting to note that neither assay was capable of detecting dihydrostreptomycin, lincomycin or novobiocin at Australian maximum residue limits and further, that the BSDA was unable to detect oleandomycin or lincomycin up to the maximum concentration used in this study. For dihydrostreptomycin, neomycin and oxytetracycline HCl, the published values for the BSDA showed a wide range in the minimum detectable concentrations. Because of this, the BSDA test for these three antibiotics was omitted in this study.

The sensitivity of *B. stearothermophilus* to \(\beta\)-lactam antibiotics is shown in Figure 3.1. Both the BSDA and the Delvo SP assays were able to detect ampicillin below concentrations which would cause inhibition of the starter cultures used. No measured change in end point pH was noted in the cultures containing the highest concentrations assessed when compared to their respective controls. This, and the detection data for penicillin G (Figure 3.2),
cloxacillin (Figure 3.3) and cefuroxime sodium (Figure 3.4) confirm the reported sensitivity of antibiotic assays using *Bacillus stearothermophilus* to detect β-lactam antibiotics.
Table 3.1: Comparison of minimum antibiotic concentrations detectable by the BSDA and Delvo SP screening systems and maximum residue limits (MRL).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>BSDA $^1$</th>
<th>Delvo SP $^1$</th>
<th>MRL $^{1,4}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>0.005</td>
<td>0.0025</td>
<td>0.01</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>n/a</td>
<td>0.0015</td>
<td>0.0025</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>n/a</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>n/a</td>
<td>0.0032</td>
<td>0.1</td>
</tr>
<tr>
<td>Dihydrostreptomycin</td>
<td>4-13$^2$</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Neomycin</td>
<td>1-22$^2$</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Oxytetracycline HCl</td>
<td>0.15-0.5$^2$</td>
<td>0.16</td>
<td>0.1$^5$</td>
</tr>
<tr>
<td>Oleandomycin</td>
<td>&gt;4.0$^3$</td>
<td>0.1</td>
<td>0.1$^6$</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>&gt;1.0$^3$</td>
<td>0.2</td>
<td>0.02</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>0.2</td>
<td>0.5</td>
<td>0.1</td>
</tr>
</tbody>
</table>

1. Antibiotic concentration in µg ml$^{-1}$.
2. Published value from IDF Bulletin 258.
3. No detection up to the maximum concentration tested.
5. Tetracyclines.
Figure 3.1: *L. lactis* strains incubated in the presence of ampicillin.

Activity of *L. lactis* subsp. *cremoris* strain ASCC 47, *L. lactis* subsp. *cremoris* strain ASCC 92 and *L. lactis* subsp. *lactis* strain ASCC 519 grown in the presence of ampicillin. Assay conditions are described in Materials and Methods. Values are means of pH end point determinations from experiments carried out in triplicate. Vertical bars span +/- 2 SD. Downward pointing arrows indicate experimental Delvo SP detection limit. Upward pointing arrows indicate experimental BSDA detection limit.
Figure 3.2: *L. lactis* strains incubated in the presence of penicillin G.

Activity of *L. lactis* subsp. *cremoris* strain ASCC 47, *L. lactis* subsp. *cremoris* strain ASCC 92 and *L. lactis* subsp. *lactis* strain ASCC 519 grown in the presence of penicillin G. Assay conditions are described in Materials and Methods. Values are means of pH end point determinations from experiments carried out in triplicate. Vertical bars span +/- 2 SD. Antibiotic concentrations are given in µg ml⁻¹. Downward pointing arrows indicate experimental Delvo SP detection limit.
Figure 3.3: *L. lactis* strains incubated in the presence of cloxacillin.

Activity of *L. lactis* subsp. *cremoris* strain ASCC 47, *L. lactis* subsp. *cremoris* strain ASCC 92 and *L. lactis* subsp. *lactis* strain ASCC 519 grown in the presence of cloxacillin. Assay conditions are described in Materials and Methods. Values are means of pH end point determinations from experiments carried out in triplicate. Vertical bars span +/- 2 SD. Antibiotic concentrations are given in µg ml⁻¹. Downward pointing arrows indicate experimental Delvo SP detection limit.
Figure 3.4: *L. lactis* strains incubated in the presence of cefuroxime.

Activity of *L. lactis* subsp. *cremoris* strain ASCC 47, *L. lactis* subsp. *cremoris* strain ASCC 92 and *L. lactis* subsp. *lactis* strain ASCC 519 grown in the presence of cefuroxime sodium Assay conditions are described in Materials and Methods. Values are means of pH end point determinations from experiments carried out in triplicate. Vertical bars span +/- 2 SD. Antibiotic concentrations are given in µg ml⁻¹. Downward pointing arrows indicate experimental Delvo SP detection limit.
As with the β-lactams, dihydrostreptomycin (Figure 3.5) and neomycin (Figure 3.6) can be detected by the Delvo SP at concentrations below those which caused inhibition to the starter cultures used in the investigation. However, the BSDA was not used to test for dihydrostreptomycin and neomycin because published detection levels of the assay show high variability in sensitivity to these antibiotics (Table 3.1).

The remaining antibiotics investigated in this study were detectable by the Delvo SP. The Delvo SP assay was able to detect oxytetracyline HCl, but the highest undetected concentration inhibited the starter cultures used in the study (Figure 3.7). Oxytetracycline HCl inhibited acid production of *L. lactis* subsp. *cremoris* strains ASCC 47 and 519 at 0.08 µg ml⁻¹, a concentration below the detectable limit of the Delvo SP assay (0.16 µg ml⁻¹). The mean end point pH measurements of both strains were significantly affected (p<0.05, p<0.01 respectively), at 0.22 units higher in the cultures containing antibiotic when compared to their respective controls. Strain 92 was also significantly inhibited (p<0.01) at the same antibiotic concentration where the mean end point pH measurement was 0.17 units higher than the control. As with dihydrostreptomycin and neomycin, the BSDA was not used to test for oxytetracyline HCl due to published variability in detection levels.

Oleandomycin and lincomycin both inhibited the starter cultures at concentrations below those detected by the Delvo SP (Figure 3.8 and 3.9). Oleandomycin significantly inhibited (p<0.01) acid production in strain 47 at concentrations of 0.25 µg ml⁻¹, which is below the minimum detection level of the Delvo SP assay. Strain 519 was also significantly inhibited (p<0.01) at the same concentration, exhibiting a mean end point pH 0.1 unit higher than the control. However, the BSDA method did not detect oleandomycin up to the maximum concentration tested (4.0 µg ml⁻¹).

Strain 47 was significantly inhibited (p<0.01) by lincomycin, where the mean end point pH was 0.15 units higher than the control at concentrations less than the minimum detection
limit (0.2 µg ml⁻¹) of the Delvo SP assay. Again, the BSDA method was not able to detect lincomycin concentrations up to the maximum used in this study (1.0 µg mL⁻¹), 5 times the minimum detection limit of the Delvo SP assay.

In the case of novobiocin, the starter cultures were inhibited at concentrations below those detectable by either of the assays used in this study (Figure 3.10). Novobiocin significantly inhibited (p<0.01) all three strains at concentrations below the detectable limit (0.5 µg ml⁻¹) of the Delvo SP assay. At 0.4 µg ml⁻¹ the mean end point pH was higher by >0.3 units for strain 47 and >0.15 units for 92 and 519 relative to their respective controls. Even at a concentration of 0.1 µg ml⁻¹, undetected by the BSDA, strain 47 was significantly inhibited (p<0.05) with a mean end point pH increase of 0.097 units relative to the control. When acid production by the same strain was continuously monitored over time in the presence of novobiocin at 0.2 and 0.4 µg ml⁻¹, it took 30 and 90 minutes (10 and 30%) longer respectively to achieve the same end point pH as the control (Figure 3.11). The novobiocin concentrations used were below the detectable limits of the Delvo SP test system although the lower antibiotic concentration used was a threshold detectable level of the BSDA assay, as determined from figure 3.10.
Figure 3.5: L. lactis strains incubated in the presence of dihydrostreptomycin.

Activity of L. lactis subsp. cremoris strain ASCC 47, L. lactis subsp. cremoris strain ASCC 92 and L. lactis subsp. lactis strain ASCC 519 grown in the presence of dihydrostreptomycin.

Assay conditions are described in Materials and Methods. Values are means of pH end point determinations from experiments carried out in triplicate. Vertical bars span +/- 2 SD.

Antibiotic concentrations are given in µg ml⁻¹. Downward pointing arrows indicate experimental Delvo SP detection limit.
Neomycin (µg ml⁻¹)

![Graph showing pH values for different L. lactis strains incubated in the presence of neomycin.]

Figure 3.6: *L. lactis* strains incubated in the presence of neomycin.

Activity results of *L. lactis* subsp. *cremoris* strain ASCC 47, *L. lactis* subsp. *cremoris* strain ASCC 92 and *L. lactis* subsp. *lactis* strain ASCC 519 grown in the presence of neomycin.

Assay conditions are described in Materials and Methods. Values are means of pH end point determinations from experiments carried out in triplicate. Vertical bars span +/- 2 SD. Antibiotic concentrations are given in µg ml⁻¹. Downward pointing arrows indicate experimental Delvo SP detection limit.
Figure 3.7: L. lactis strains incubated in the presence of oxytetracycline HCl.

Activity results of L. lactis subsp. cremoris strain ASCC 47, L. lactis subsp. cremoris strain ASCC 92 and L. lactis subsp. lactis strain ASCC 519 grown in the presence of oxytetracycline HCl. Assay conditions are described in Materials and Methods. Values are means of pH end point determinations from experiments carried out in triplicate. Vertical bars span +/- 2 SD. Antibiotic concentrations are given in µg ml⁻¹. Downward pointing arrows indicate experimental Delvo SP detection limit.
Figure 3.8: *L. lactis* strains incubated in the presence of oleandomycin.

Activity results of *L. lactis* subsp. *cremoris* strain ASCC 47, *L. lactis* subsp. *cremoris* strain ASCC 92 and *L. lactis* subsp. *lactis* strain ASCC 519 grown in the presence of oleandomycin. Assay conditions are described in Materials and Methods. Values are means of pH end point determinations from experiments carried out in triplicate. Vertical bars span +/- 2 SD. Downward pointing arrows indicate experimental Delvo SP detection limit.
Figure 3.9: *L. lactis* strains incubated in the presence of lincomycin.

Activity results of *L. lactis* subsp. *cremoris* strain ASCC 47, *L. lactis* subsp. *cremoris* strain ASCC 92 and *L. lactis* subsp. *lactis* strain ASCC 519 grown in the presence of lincomycin.

Assay conditions are described in Materials and Methods. Values are means of pH end point determinations from experiments carried out in triplicate. Vertical bars span +/- 2 SD. Downward pointing arrows indicate experimental Delvo SP detection limit.
Figure 3.10: *L. lactis* strains incubated in the presence of novobiocin.

Activity of *L. lactis* subsp. *cremoris* strain ASCC 47, *L. lactis* subsp. *cremoris* strain ASCC 92 and *L. lactis* subsp. *lactis* strain ASCC 519 grown in the presence of novobiocin. Assay conditions are described in Materials and Methods. Values are means of pH end point determinations from experiments carried out in triplicate. Vertical bars span +/- 2 SD. Downward pointing arrows indicate experimental Delvo SP detection limit. Upward pointing arrows indicate experimental BSDA detection limit.
Figure 3.11. Continuous pH monitoring of *L. lactis* subsp. *cremoris* strain 47 incubated in the presence of novobiocin.

Activity of *Lactococcus lactis* subsp. *cremoris* strain ASCC 47 grown in RSM and novobiocin at 32°C. Assay conditions are described in Materials and Methods and the data collected using a calibrated continuous pH monitoring system. The novobiocin concentrations were: (Ο) 0 µg mL⁻¹, (●) 0.2 µg mL⁻¹, (Δ) 0.4 µg mL⁻¹. An uninoculated control included in the assay remained at pH 6.4 +/- 0.05 throughout the incubation period (data not shown).
3.13 Discussion

Microbial screening tests for the detection of antibiotics in milk based on *B. stearothermophilus* are generally more sensitive to β-lactam antibiotics than to other groups of antibiotics (Honkanen-Buzalski and Reybroeck 1997). In this study, the Delvo SP assay detected the β-lactam antibiotics penicillin g, ampicillin, cefuroxime and cloxacillin at levels below that which would cause significant inhibition of the starter cultures assayed. The Delvo SP assay was also sufficiently sensitive to detect dihydrostreptomycin and neomycin below concentrations that would significantly inhibit the starters.

The minimum concentrations of cloxacillin, dihydrostreptomycin, lincomycin, novobiocin, oleandomycin and oxytetracycline HCl detectable by the Delvo SP assay were higher than the Australian MRL. Further, published BSDA detection values for dihydrostreptomycin, neomycin and oxytetracycline HCl indicate a wide concentration range between “all negative” and “all positive” responses (Heeschen *et al.* 1991) which suggests that the assay is unsuitable for the detection of residues of these antibiotics.

Acid production by starters was not affected by ampicillin at concentrations up to 0.05 µg ml⁻¹ (50ppm) which is 20 and 10 times higher than the concentration detected by the Delvo SP and the BSDA test systems respectively. No significant reduction in acid production by any of the three starters tested was observed at or below the detectable concentrations of penicillin g, while cloxacillin did not inhibit the starters even at concentrations which were at least double those detectable by the Delvo SP assay. The Delvo SP assay was also able to detect the β-lactam cefuroxime below concentrations that were inhibitory for the starter cultures and below the Australian MRL. The results from this study validate the use of *B. stearothermophilus* as an antibiotic detection organism and, more specifically, the Delvo SP assay for detecting β-lactam antibiotics in milk. Therefore, its continued use is important as a
DFSV survey of veterinary practices indicated that β-lactams form 27% of antibiotic dispensations for intramammary lactating cow mastitis therapies (Long 2001).

Dihydrostreptomycin, an aminoglycoside, was detectable by the Delvo SP assay at a concentration below that which caused inhibition of the three starter cultures. However, published values for the detection of dihydrostreptomycin by the BSDA, which range from 4-13 µg ml⁻¹, indicate uncertainty in detecting this compound in milk. The Delvo SP assay successfully detected neomycin at 0.4 µg ml⁻¹, five times lower than the maximum concentration investigated in this trial where no starter inhibition was observed for two of the three strains. Published values for neomycin detection by the BSDA range from 1-22 µg ml⁻¹ and consequently the assay may be unsuitable since a DFSV survey has indicated that aminoglycosides (neomycin and dihydrostreptomycin) form a large proportion (approximately 30% by frequency) of antibiotic dispensations in Australia (Long 2001).

The minimum detection limit of the Delvo SP assay for oxytetracycline HCl determined in this study is the same as the published all negative values for the BSDA method (Heeschen et al. 1991). Importantly, the BSDA was unable to detect either oleandomycin or lincomycin at the maximum concentrations used in these trials. These macrolides account for over 13% (by frequency) of the intramammary lactating cattle antibiotics dispensed in Australia (Long 2001).

The BSDA procedure can detect novobiocin at 0.2 µg ml⁻¹, at less than one half the minimum detection level of the Delvo SP assay. Although a novobiocin concentration of 0.1 µg ml⁻¹ did not inhibit two of the three strains sufficiently to alter the mean end point pH ≥ 0.1 relative to their respective controls, novobiocin residues in the 0.1 to 0.2 µg ml⁻¹ range may cause some difficulties with starter activity. Furthermore, the BSDA was more sensitive than the Delvo SP assay for the detection of novobiocin, which is in contrast to results for the other antibiotics. According to Mason et al. (2001), novobiocin use has increased recently.
and in the DFSV survey it was reported to be present in just over 12% of lactating cow mastitis therapies dispensed nationally (Long 2001).

From this study it is evident that a number of non-\( \beta \)-lactam antibiotics, in particular novobiocin, are able to inhibit starter culture performance to some extent at concentrations below those detectable by the Delvo SP and the BSDA screening assays. While the extent of inhibition may appear to be small at very low residue concentrations, the effects may still be sufficient to interfere with cheese manufacture.
“Enzymic hydrolysis of milk lipids to free fatty acids and partial glycerides has both beneficial and detrimental effects...”

Deeth and Fitzgerald, 1983

4.0 Acid production by a *L. lactis* in temperature abused milk

4.1 Dairy product manufacture

In the modern context, where primary producers are often separated from consumers by large distances, delivery of dairy products poses considerable technological challenges. Milk must be sanitarily removed from the animal, transported to factories, stored at the factory site, heated to kill pathogenic organisms and often, standardised for fat and protein concentrations. Further processing such as fermentation, blending with other ingredients, packaging and distribution all vary according to the specific product. While the objective is to add product value, each step in the process presents its own challenge in maintaining and enhancing product safety. The implementation of bulk tank refrigeration on the farm greatly improved the industry’s capability of delivering safe dairy products through increasingly larger production and distribution systems.

Raw milk refrigeration on the farm minimises the growth of adventitious organisms, the initial population of which is usually quite small. Maintenance of the “cold chain” thereafter is a fundamental requirement of quality control measures. However, extended storage at refrigeration temperatures prior to processing leads to changes in various milk components. These effects have been extensively investigated with regards to product quality (Law 1979).


### 4.2 Refrigerated storage of raw milk

The activities of naturally occurring lipolytic and proteolytic enzymes in raw milk, as well as the growth of any microorganisms that may be present, are reduced but not eliminated at refrigeration temperatures. Refrigeration exerts a selective pressure on the raw milk microflora benefiting psychrotrophs, or organisms capable of growth at these temperatures. Psychrotrophs contribute to the lipolytic and proteolytic processes in refrigerated milk by the release of enzymes, the effects of which have been extensively studied and reviewed.

Cousin (1982), in a study of microbial growth at refrigeration temperatures, showed that numbers of *Pseudomonas fragi* and *Pseudomonas fluorescens* doubled at 4°C in 5.5 and 6.7 hours respectively. Celestino *et al.* (1996) confirmed the increase in cell number and proportion of the microbial population of psychrotrophic bacteria in refrigerated milk stored at 4°C for 48 hours. Garg (1990) discussed the development of off-flavours in a variety of dairy products as a result of the activity of lipolytic and proteolytic enzymes secreted by psychrotrophs. Shaw (1994) also discussed the technical difficulties associated with the presence of psychrotrophs in cheese production, including the development of rancidity, other off flavours and the inhibition of diacetyl production by starter bacteria.

Rowe and Gilmore (1985) highlighted the limitations of current methods employed for hygiene assessment and shelf life prediction since typical psychrotrophic organisms are unable to reduce methylene blue and resazurin. In support of this view, Sørhaug and Stepaniak (1997) focused on the need to develop rapid and sensitive assays for predicting shelf life based on the activity of selected enzymes known to be secreted by psychrotrophs.

The growth of pathogenic organisms at refrigeration temperatures is a cause for greater concern to the consumer. Champagne *et al.* (1994) reviewed the health implications of food borne disease linked to dairy products made from pasteurised milk and subsequently stored at 4°C. Under these conditions, post-pasteurisation contamination by *Listeria* sp. is the most
important consideration. However, in spite of the serious problems arising through the necessity of refrigerated storage, some positive effects due to psychrotrophic activity in milk are evident.

Harishrinivas and Shankar (1997) prepared yoghurts with milks pre-cultured with lipase and proteinase positive and negative cultures of *Pseudomonas* spp. They found that the yoghurts prepared in the enzyme positive milks yielded higher cell counts, titratable acidity, tyrosine values, total FFA and improved acceptability scores. Lactic acid production by *Lactococcus lactis* subsp. *lactis* and subsp. *cremoris* was greater in milks pre-cultured by added psychrotrophs (Cousin and Marth 1977a). Jaspe *et al.* (1995) also found increased lactate production by an LAB strain when grown simultaneously with a *Pseudomonas* strain when inoculated at a $10^6:10^5$ cfu ml$^{-1}$ ratio respectively.

### 4.3 Lipolysis

The highest incidence of lipolytic activity among the psychrotrophic Gram-negative flora of commercial raw milks was found in strains of *Pseudomonas fluorescens* and *Pseudomonas fragi* (Law and Chapman 1976). The same workers demonstrated the development of rancidity in Cheddar cheese made with milk pre-cultured with similar organisms. Shelley *et al.* (1987) examined thirty-six raw milk samples for lipolytic organisms. From these 205 strains capable of extensive lipolytic activity were isolated, 63.9% of which were *Pseudomonas fluorescens* and 31.2% were *Pseudomonas fragi*. Dieckelmann *et al.* (1998) noted the diversity of lipolytic enzymes among *Pseudomonas* spp. In addition to affecting raw milk, psychrotrophs can also cause problems in pasteurised milk. Lipase from *Pseudomonas* spp. remains active after pasteurization (Deeth and Fitzgerald 1983) while *Pseudomonas* spp. dominate in post-pasteurization milk (Craven and MacCauley 1992).

Apart from psychrotrophic enzymatic processes, free fatty acid concentrations in raw milk may be affected by mechanical handling. For example milking machine design has been
shown to significantly affect raw milk FFA (Evers and Palfreyman 2001). The natural lipases also present in raw milk can contribute to the degradation of the milk fat. The various factors contributing to native milk lipase activity and the concomitant increase in FFA have been reviewed by Anderson (1983).

### 4.3.1 Starter culture inhibition

Tarassuk and Smith (1940) attempted to identify the inhibitory effects on acid development by *Streptococcus (Lactococcus) lactis* in milk made rancid by the action of native lipase. They attributed the inhibition to the effects of reduced surface tension. In a later study, Costilow and Speck (1951) identified three medium chain length fatty acids (caprylic, capric and lauric) as being the most inhibitory to the growth of *S. (Lactococcus) lactis*, while short chain (butyric, caproic) and long chain fatty acids (palmitic, stearic, oleic, linoleic and linolenic) showed no inhibitory effect. Anders and Jago (1964a) showed that oleic acid inhibited *Streptococcus (Lactococcus) subsp. cremoris* strain C13, although resistance to inhibition by oleic acid was also noted in a variant of the same strain (Anders and Jago 1964b). Schwab (1969) reported inhibition of thermophilic cultures, again in rancid milk, due to the effect of elevated lauric acid concentrations. These fatty acids are believed to affect cell membrane permeability, interfering with the cell’s capacity to maintain a pH gradient (Desmazeaud 1996).

### 4.4 Proteolysis

Milk proteins are basically comprised of caseins which constitute approximately 80% of the total protein in milk, while whey proteins account for the remainder. Caseins are the most susceptible to degradation during microbial growth (Cousin and Marth 1977b). The degradation products consist principally of small peptides and amino acids which can then be utilised by microorganisms. *Pseudomonas* spp. possess strong proteolytic systems that are
resistant to heat denaturation and the casein content and pH of normal milk facilitate their action (Adams et al. 1975).

4.4.1 Starter culture stimulation

*Lactococcus lactis* has complex nutritional requirements and depends on casein hydrolysis to support growth in milk. Casein hydrolysis is achieved by a proteinase associated with the cell envelope. An ATP dependent oligopeptide transport system, as well as di- and tripeptide transport systems, facilitate cellular uptake of the casein derived peptides. Intracellular peptidases further degrade these peptides into amino acids. The cell envelope associated proteinase occurs in two major forms, referred to as PI and PIII, however there are a number of intermediate forms. PI primarily hydrolyses β-casein, while PIII is capable of hydrolysing αs1-, β- and κ-casein (Fox et al. 2000). It has been shown that the growth of *Streptococcus (Lactococcus) lactis* is stimulated when grown in milk that has been pre-cultured for five days at 7°C with a *Pseudomonas* sp. isolated from raw milk (Cousin and Marth 1977a). In this case the protein degradation products resulting from the proteolytic activities of the *Pseudomonas* sp. have been utilised by the starter organism.

4.5 Aim of present study

Extended milk storage at refrigeration temperatures facilitates the growth of psychrotrophic organisms and the production of extracellular lipases. These lipases, together with the natural lipases present in milk, can degrade the milk lipids forming free fatty acids that may inhibit cheese starter bacteria. In contrast, growth may be stimulated due to increased availability of peptides and amino acids liberated by the psychrotrophic proteinases. The aim of this section was to determine the effect on the activity of a commercial starter culture grown in milk which had previously been temperature abused with the consequent growth of two commonly isolated *Pseudomonas* spp.
4.6 Results

The two psychrotrophic organisms, *Pseudomonas fragi* strain 71 and *Pseudomonas fluorescens* strain 113, were grown in pasteurised whole milk for up to 6 days at 7°C (Figure 4.1). Cell numbers for both organisms approached $10^5$ cfu/ml at 24 hours and achieved maximal populations in the six day period. As the cell numbers achieved after one day were higher than the average raw milk plate counts that major Victorian milk processors routinely record (Mitchell, pers. comm.) the 6 day incubation period at 7°C represents an extreme case of psychrotrophic contamination. Despite the growth of these organisms, no increase in proteolytic by-product availability was measured in the 6 day storage period from either organism (Figure 4.2). Further, by day six only the *P. fragi* had produced a measurable increase in available free fatty acids (Figure 4.3). When the activity of *L. lactis* subspecies *cremoris* strain ASCC 47 was measured during growth in the pre-cultured milks, no significant changes were observed when compared to respective controls (Figure 4.4).
Figure 4.1: Cell counts of psychrotrophic organisms in PWM.

Cell counts of *Pseudomonas fragi* strain 71 and *Pseudomonas fluorescens* strain 113 when grown in pasteurised whole milk at 70°C. Cell numbers were determined as described in Materials and Methods. Horizontal bars represent the mean of triplicate determinations. Vertical bars represent +/- 2 SD.
Figure 4.2: TCA-soluble nitrogen in pre-cultured PWM.

TCA-soluble nitrogen concentrations in pasteurised whole milk during the growth of *Pseudomonas fragi* strain 71 and *Pseudomonas fluorescens* strain 113 incubated for 6 days at 70°C. Determination of TCA-soluble nitrogen available in daily samples was carried out as described in Materials and Methods.
Figure 4.3: Total free fatty acids in pre-cultured PWM.

Total free fatty acids made available in pasteurised whole milk during the growth of

*Pseudomonas fragi* strain 71 and *Pseudomonas fluorescens* strain 113 incubated for 6 days at

7°C. Total free fatty acids were determined in daily samples as described in Materials and

Methods.
Figure 4.4: Activity of a *L. lactis* in pre-cultured PWM.

End point pH achieved by *L. lactis* subsp. *cremoris* strain ASCC 47 in pasteurised whole milk pre-cultured at 7°C for up to 6 days with *Pseudomonas fragi* strain 71 and *Pseudomonas fluorescens* strain 113. Activity of the starter culture was determined as described in Materials and Methods.
4.7 Discussion

Tarassuk and Smith (1940) noted changes in starter behavior in milks that had been made rancid, while Cousin and Marth (1977a) held the pre-cultured milks at 7°C for 5 days before conducting their assays. These observations therefore, could readily be explained if they were noted in a production environment. Therefore, for the purposes of this section of the study, any detrimental effect to starter culture activity should be observed prior to the development of obvious milk quality defects.

Rapid elevation in the total plate count of both milks occurred over the trial period. Although there are no regulations regarding total plate count limits in raw milk, major dairy processors in Victoria, Australia, routinely expect counts to be $2 \times 10^4$ cfu ml$^{-1}$ or lower, (Mitchell, pers. comm.). However, it is possible that the milks could still be used at this point as data on the cell count of the milk would not be available immediately.

No proteolysis is measurable within the assay period and lipolytic effects are not observed until the sixth day. McKay and Beacham (1995) demonstrated that fatty acid accumulation depended on the balance of fatty acid production due to lipolysis and fatty acid consumption, both of which were dependant on incubation temperature. It is possible therefore, that any inhibitory compounds liberated by fat deterioration under these conditions were concurrently metabolised. This consideration could also apply to the proteolytic degradation products. However, the deleterious cheese quality effects reported in the literature would be observed during maturation if this milk was actually used in cheese manufacture.

The activity of the starter culture used in the study was not adversely affected by the pre-culturing by either of the *Pseudomonas* species. If the acid production by the starter culture had been inhibited, cheese makers would have an early indication of deleterious quality effects prior to the product entering costly maturation storage.
“...bovine milk contains several anti-microbial agents which are beneficial to the calf...”

Robert G. Jensen, 1995

5.0 Sensitivity of *L. lactis* strains to a model LPS

5.1 Native antimicrobial systems

Bovine milk provides for the nutritional needs of the calf as well as conferring passive immunity. The immunological factors included in milk may also contribute to the defenses of the mammary gland (Leigh *et al.* 1990, Collins *et al.* 1988). Thus, while milk is a suitable medium for fermentation by lactic acid bacteria, these microbiological defense systems may impede the growth of these and other organisms (IDF 1991, Reiter 1978).

The immunological properties of milk may be divided into specific and nonspecific functions. Immunoglobulins, which in milk are primarily of the IgG type, bind to specific antigens. Nonspecific immune defenses depend mainly on lactoferrin (LF), lysozyme (LYS), lactoperoxidase (LPS) and hydrogen peroxide generated by various oxidases present in milk (Reiter 1976).

5.2 The lactoperoxidase system

5.2.1 Lactoperoxidase

The three part lactoperoxidase system (LPS) is made up of the 77.5 kDa enzyme lactoperoxidase (EC 1.11.1.7), the thiocyanate ion and hydrogen peroxide (Reiter and Härnulv 1984). Lactoperoxidase is similar in structure to human myeloperoxidase, eosinophil peroxidase and thyroperoxidase (Cals *et al.* 1991). The antimicrobial activity of
lactoperoxidase may be enhanced when found in conjunction with other nonspecific enzymes such as lysozyme (EC 3.2.1.17) (Hulea et al. 1989). Rennet whey from bovine milk contains approximately 35 mg l\(^{-1}\) lactoperoxidase (Ye et al. 2000) which in milk retains about 70% activity after HTST (72\(^{\circ}\)C/15 sec.) pasteurisation (Barrett et al. 1999). Thus, the availability of lactoperoxidase is not a limiting factor for the system to function in the cheese vat.

5.2.2 Thiocyanate

Thiocyanate (SCN-) concentrations in milk vary according to diet, the primary source being the detoxification of cyanogenic compounds from plants of the *Brassicaceae* family (Tupper and Reay 1973). Bulked milk from three collection regions in northern Victoria, Australia were assessed for thiocyanate concentrations, which ranged from 1.3 to 8.1ppm (Roginski et al. 1984a). In comparison, a survey of milk from 64 individual farms in West Gippsland, Victoria, Australia, determined thiocyanate concentrations ranging from 7.1 to 13.3ppm, with an average of 10.2ppm. The same study noted that elevated thiocyanate levels may have a negative impact on Cheddar cheese manufacturing as milk from cows fed more than 4 kg per day of turnips was found to inhibit a Cheddar cheese starter culture (Moate et al. 1996).

5.2.3 Hydrogen peroxide

Hydrogen peroxide, the third component of the LPS, may exist in low concentrations in freshly drawn milk (Reiter 1976). The presence of hydrogen peroxide in milk may be due to the metabolic functions of oxidases such as xanthine oxidase (EC 1.1.3.22), which has a broad substrate specificity (Björck and Claesson 1979). However, catalase (EC1.11.1.6), also present in milk, may remove hydrogen peroxide (Kitchen et al. 1970). Thus, for the activation of the lactoperoxidase system, a source of hydrogen peroxide will cause an antibacterial effect that is proportional to the thiocyanate present (Björck 1978).
5.3 Formation of H$_2$O$_2$ by lactic acid bacteria

Generally, microorganisms must contend with the presence of oxygen, whether or not they possess aerobic metabolic functions. In LAB, flavoprotein oxidase enzymes are responsible for the direct interaction with oxygen leading to its detoxification. Molecular oxygen (O$_2$) can be reduced by an NADH oxidase to hydrogen peroxide (H$_2$O$_2$) while H$_2$O$_2$ can be further reduced by an NADH peroxidase (EC1.11.1.1) to yield water (Anders et al. 1970). Therefore the accumulation of H$_2$O$_2$ is a result of the balance between the processes that produce it and those that eliminate it. In some cultures exposed to oxygen, H$_2$O$_2$ accumulates to inhibitory levels (Condon 1987). Premi and Bottazzi (1972) found that of the LAB species examined, *Lactobacillus lactis* produced the greatest quantities of H$_2$O$_2$ however, no correlation was observed with either aerobic or anaerobic growth and H$_2$O$_2$ accumulation in a range of LAB (Whittenbury 1964). Sakamoto and Komagata (1996) investigated the aerobic growth of 22 LAB strains including *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Streptococcus* and *Enterococcus* spp., identifying some that produced H$_2$O$_2$. *Lactobacillus debrueckii* subsp. *bulgaricus* reduced O$_2$ to H$_2$O$_2$ with an NADH oxidase, presumably to eliminate the oxygen present although this detoxification of oxygen leads to an overproduction of H$_2$O$_2$ which can also cause oxidative stress (Marty-Teysset et al. 2000).

*Lactobacillus fermentum* H3, *Lb. fermentum* Y6 and *Lb. delbrueckii* subsp. *delbrueckii* NRIC 1053, did not grow well and accumulated H$_2$O$_2$ in the defined culture medium under ordinary aerobic conditions. However, their growth was gradually enhanced by adding peroxidase up to 1U ml$^{-1}$ (Sakamoto et al. 1998). H$_2$O$_2$ accumulated to a concentration of over 20 µM during growth of *Streptococcus thermophilus* in UHT milk. Addition of normal biological concentrations of lactoperoxidase to UHT milk caused inhibition of the starter organism. These authors also concluded that seasonal inhibition of *S. thermophilus* is
possible due to increased concentrations of lactoperoxidase in post-calving, late winter-spring milk (Nicol et al. 1995).

Aerating cultures of *Streptococcus* (*Lactococcus*) *lactis* C10 led to H$_2$O$_2$ accumulation in synthetic media. When the energy source was galactose, lactose or maltose, H$_2$O$_2$ reached auto-inhibitory levels (Grufferty and Condon 1983). The formation of H$_2$O$_2$ by lactococci was found to occur through the action of a reduced nicotinamide adenine dinucleotide (NADH) oxidase which catalyzed the oxidation of NADH by molecular oxygen. The enzyme was activated by flavine adenine dinucleotide (FAD). Some of the H$_2$O$_2$ formed was removed through the action of an NADH peroxidase (EC 1.11.1.1) (Anders et al. 1970). Zitzelsberger et al. (1984) tested 23 strains of streptococci, finding that only 7 strains had NADH peroxidase activity, and that the strain of *Streptococcus* (*Lactococcus*) *lactis* tested was negative for its activity.

### 5.4 Antibacterial mechanisms and inhibitory effects of LPS

The lactoperoxidase system has apparent maximal activity 3-4 days after milking (Ravanis and Lewis 1995). Hypothiocyanite (OSC${\text{N}}^{-}$) is the reactive compound transiently generated by the LPS (Modi et al. 1991, Pruitt et al. 1982) which has been shown to oxidise sulphhydryl groups (Thomas and Aune 1978). Although the mode of action is still unclear, a number of physiological effects have been noted in organisms grown in its presence, principally the leakage of K$^+$ and amino acids and the inhibition of carbohydrate and amino acid uptake (Condon 1987). An activated LPS has been shown to irreversibly inhibit *Escherichia coli* (Thomas and Aune 1978) while *Staphylococcus aureus* numbers were reduced by two log values in its presence (Kangumba et al. 1997). Gram-negative, catalase positive organisms such as *Salmonella* spp. (Purdy et al. 1983) and *Pseudomonas* spp. are also inhibited (Björck 1978), as is the growth of some fungi (Benoy et al. 2000). Total inhibition of *Aeromonas hydrophila* was also evident when the LPS was activated in...
pasteurised ewes’ milk (Santos et al. 1995) while acid production and or glucose metabolism by pathogenic and oral streptococcal species is also inhibited (Loimaranta 1998, Mickelson and Brown 1985, Mickelson 1977).

Siragusa and Johnson (1989) and Gaya et al. (1991) noted extended lag periods for Listeria monocytogenes at refrigeration temperatures in the presence of the LPS, while Kamau et al. (1990) found that a Listeria sp. was more sensitive to heat treatments in milk that had previously been activated for the LPS. Listeria spp. were also more sensitive to bacteriocins when cultured at 8°C in the presence of the LPS (Rodriguez et al. 1997; Zapico et al. 1998). Further, Ravishankar and Harrison (1999) showed that acid adaptation of three strains of L. monocytogenes did not provide any survival benefit for their growth in the presence of the LPS.

An activated LPS can inhibit oral streptococci up to 500 times more effectively than that caused by H₂O₂ alone (Thomas et al. 1994). In the absence of the other functional components of the LPS, 150 μM H₂O₂ is reported to only slightly inhibit acid production by lactic streptococci (Subramanian and Olson 1967), whereas inhibition due to a functional LPS can be achieved by the addition of only 100 μM H₂O₂ (Roginski et al. 1984a). The glycolytic enzyme hexokinase (EC 2.7.1.2) was totally inhibited when assayed in the presence of the complete LPS (Oram and Reiter 1966). The LPS also inhibits native lipoprotein lipase, thus alleviating lipolysis in milk (Ahrne and Björck 1985). As a result of its antibacterial activity, an activated LPS has been shown to be effective as a preservation method for raw milk in regions where refrigeration is not available (Haddadin et al. 1996, Wolfson and Sumner 1993, IDF 1983).
5.5 Variations in starter inhibition due to LPS

Variations in acid production by \textit{L. lactis} strains when grown in HTST “cheese milk” (pasteurized at 72°C for 15 seconds) vs. “steamed milk” (95°C for 30 minutes) were attributed to the inhibition caused by an active LPS remaining in the HTST milk (IDF 1991). Oram and Reiter (1966) demonstrated that \textit{Streptococcus (Lactococcus) lactis} strain 803 was resistant to the LPS and noting that some strains of lactococci may possess a “reversal factor”. Conversely, some phage-resistant derivatives (mutants) of \textit{L. lactis} strains were more susceptible to H$_2$O$_2$ and the LPS than were their parent organisms (Roginski et al. 1991). Moreover, inhibition of thermophilic starters is reported to be strain dependent and to vary with the strain’s ability to produce H$_2$O$_2$ (Guirguis and Hickey 1987).

5.6 Aim of present study

The objective of this section was to investigate the extent of strain dependent sensitivity to an activated LPS, to determine the metabolic differences between sensitive and insensitive strains and to confirm the inhibitory effects of the LPS on hexokinase activity in physiologically intact cells.

5.7 Results

Nine \textit{Lactococcus lactis} subsp. \textit{cremoris} and four \textit{Lactococcus lactis} subsp. \textit{lactis} strains were surveyed for their sensitivity to the model LPS by determining the strain’s activity in RSM containing the model LPS as described in the Materials and Methods. A range in sensitivity to the LPS was observed among the thirteen strains with no strains being stimulated by the system. Although all strains tested were significantly inhibited (p<0.01), \textit{Lactococcus lactis} subsp. \textit{lactis} strain ASCC 307 and \textit{cremoris} strain ASCC 458 were chosen to represent the less sensitive strains, while \textit{cremoris} strains ASCC 441 and ASCC 900 were chosen as the most sensitive strains (Figure 5.1).
All 13 strains were assayed for LPS sensitivity in autoclaved medium (RSM) without agitation and with minimal atmospheric surface contact, minimising dissolved oxygen, consequently reducing the likelihood of additional H$_2$O$_2$ being produced by any of the strains. In order to explain the differences in sensitivity to the LPS, *cremoris* strains 441, 458 and 900, and *lactis* strain 307 were each tested for NADH peroxidase activity. None of the strains produced measurable NADH peroxidase activity (data not shown).

*Lactococcus lactis* subsp. *cremoris* strains 458 and 900, representing the extremes in sensitivity to the model LPS (Figure 5.1) were compared for L-lactate production when grown in M17 broth with galactose as the carbon source in the presence of the model LPS. *Lactococcus lactis* subsp. *cremoris* preferentially use the PEP/PTS transport system when metabolising galactose. Galactose is phosphorylated during transport across the cell membrane and is metabolised via the tagatose pathway. In this study, no significant inhibition (p>0.05) of lactate production was observed in either strain (Figure 5.2).

*Lactococcus lactis* also utilise a PEP/PTS pathway to transport fructose into the cell which is ultimately metabolised through the glycolytic pathway. When strains 458 and 900 were assayed for L-lactate production following growth in M17 broth with fructose as the carbon source in the presence of the model LPS, again no significant inhibition (p>0.05) of either strain was observed (Figure 5.3).

However, when *cremoris* strains 458 and 900 were grown in M17 broth with lactose as the carbon source in the presence of the model LPS, both were significantly inhibited (p<0.05 and p<0.01 respectively). As with the initial survey (Figure 5.1), the more sensitive strain exhibited a greater reduction in L-lactate production, as the L-lactate produced in the LPS culture was <30% of the control, while L-lactate production by the less sensitive strain was 80% of the control (Figure 5.4). The diminished L-lactate production would suggest
inhibition of hexokinase by the activated LPS resulting in a reduced rate of glucose
(originating from lactose) phosphorylation to glucose-6-phosphate.

L-lactate production was significantly inhibited by cremoris strains 441 (p<0.01), 458
(p<0.01) and 900 (p<0.01) and lactis strain 307 (p<0.05) when grown in M17 broth
containing glucose as the carbon source in the presence of the LPS (Figures 5.5 and 5.6). The
most sensitive strains in the initial survey (Figure 5.1) were inhibited to a greater extent in the
presence of the model LPS. Mean L-lactate production by strain 900 was 41% of the control
while the less sensitive strain 458, was 80% of the control.
Figure 5.1: Survey of starter strains in RSM and LPS.

Activity of *L. lactis* strains incubated in RSM containing LPS as described in Materials and Methods. Vertical bars span +/- 2 S.D.
Figure 5.2: *L. lactis* strains 458 and 900 grown in M17 containing galactose and LPS.

L-lactate production by *Lactococcus lactis* subsp. *cremoris* strains ASCC 458 and *Lactococcus lactis* subsp. *cremoris* strain ASCC 900 in M17 broth with galactose +/- model LPS. Growth was carried out at 32°C for 5 hours and L-lactate determined as described in Materials and Methods. The blank sample was inoculated and held at 4°C until the incubation period was complete to account for any L-lactate included in the inoculum. The control culture contained no LPS.
Figure 5.3: *L. lactis* strains 458 and 900 grown in M17 containing fructose and LPS.

L-lactate production of *Lactococcus lactis* subsp. *cremoris* strain ASCC 458 and *Lactococcus lactis* subsp. *cremoris* strain ASCC 900 in M17 broth with fructose +/- model LPS. Growth was carried out at 32°C for 5 hours and L-lactate determined as described in Materials and Methods. The blank sample was inoculated and held at 4°C until the incubation period was complete to account for any L-lactate included in the inoculum. The control culture contained no LPS.
Figure 5.4: *L. lactis* strains 458 and 900 grown in M17 containing lactose and LPS.

L-lactate production of *Lactococcus lactis* subsp. *cremoris* strain ASCC 458 and *Lactococcus lactis* subsp. *cremoris* strain ASCC 900 in M17 broth with lactose +/- model LPS. Growth was carried out at 32°C for 5 hours and L-lactate determined as described in Materials and Methods. The blank sample was inoculated and held at 4°C until the incubation period was complete to account for any L-lactate included in the inoculum. The control culture contained no LPS.
Figure 5.5: *L. lactis* strains 307 and 458 grown in M17 containing glucose and LPS.

L-lactate production of *Lactococcus lactis* subsp. *lactis* strain ASCC 307 and *Lactococcus lactis* subsp. *cremoris* strain ASCC 458 in M17 broth with glucose +/- model LPS. Growth was carried out at 32°C for 5 hours and L-lactate determined as described in Materials and Methods. The blank sample was inoculated and held at 4°C until the incubation period was complete to account for any L-lactate included in the inoculum. The control culture contained no LPS.
Figure 5.6: *L. lactis* strains 441 and 900 grown in M17 containing glucose and LPS.

L-lactate production of *Lactococcus lactis* subsp. *cremoris* strain ASCC 441 and *Lactococcus lactis* subsp. *cremoris* strain ASCC 900 in M17 broth with glucose +/- model LPS. Growth was carried out at 32°C for 5 hours and L-lactate determined as described in Materials and Methods. The blank sample was inoculated and held at 4°C until the incubation period was complete to account for any L-lactate included in the inoculum. The control culture contained no LPS.
Figure 5.7: L-lactate production of *Lactococcus lactis* subsp. cremoris strain ASCC 900 in lactose +/- LPS +/- dithiothreitol. Growth was carried out at 32°C for 5 hours and L-lactate determined as described in Materials and Methods. The blank sample was inoculated and held at 4°C until the incubation period was complete to account for any L-lactate included in the inoculum. L= LPS, D= dithiothreitol
Figure 5.8: L-lactate production of *Lactococcus lactis* subsp. *cremoris* strain ASCC 900 in glucose +/- LPS +/- dithiothreitol. Growth was carried out at 32°C for 5 hours and L-lactate determined as described in Materials and Methods. The blank sample was inoculated and held at 4°C until the incubation period was complete to account for any L-lactate included in the inoculum. Materials and Methods. L= LPS, D= dithiothreitol
Dithiothreitol significantly alleviated ($p<0.05$ and $p<0.01$ respectively) the inhibition of *Lactococcus lactis* subsp. *cremoris* strain ASCC 458 and ASCC 900 when grown in M17 broth with lactose or glucose as the carbon source in the presence of the LPS (Figure 5.7, 5.8), indicating the protection of sulfhydryl groups.

PEP/PTS mediated glucose uptake was observed in both strains 458 and 900. As a control, glucose uptake by PEP depleted cells of *Lactococcus lactis* subsp. *lactis* strain ASCC 249 (ML3) was also observed, but at a much reduced rate when compared to the culture not depleted of PEP, confirming Thompson’s results. However, no measurable uptake of glucose in PEP depleted cells of either of the *cremoris* strains (458 or 900) occurred (data not shown).

*Lactococcus lactis* subsp. *lactis* strain ATCC 7962 was incubated in M17 broth with galactose as the carbon source at $32^\circ C$ containing the LPS. During log phase of growth the difference in mean $OD_{600}$ of the LPS containing culture and its control was $>0.14$ units ($p<0.01$) (Figure 5.9). The point of maximal inhibition was after seven hours of incubation where the mean $OD_{600}$ of the LPS containing culture was approximately 10% lower than the control. These results indicate that growth of strain ATCC 7962 was inhibited by the model LPS when grown on galactose. Further, the exhaustion of LPS reactants over time was demonstrated, since by the end of the assay there was no significant difference ($p>0.05$) in mean optical density between the LPS containing culture and its control (Table 5.1).

Relevant details of sugar transport and subsequent metabolic pathways for *Lactococcus lactis* have been provided in a diagram adapted from Thompson and Gentry-Weeks (1994) (Figure 5.10).
Figure 5.9: Mean optical density of *Lactococcus lactis* subsp. *lactis* strain ATCC 7962 cultures when grown in M17 containing galactose as the carbon source in the presence of the LPS incubated at 32°C as described in Materials and Methods. (•), LPS containing culture; (O), control. Experiment conducted in triplicate. Spectrophotometric measurements conducted at 600nm.
Table 5.1: Differences in means and respective p values (from t-test) of optical density measurements of *Lactococcus lactis* subsp. *lactis* ATCC 7962 when grown on galactose.

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Difference in means$^1$ (LPS- and LPS+)</th>
<th>p value$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>1</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>2</td>
<td>0.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>3</td>
<td>0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>4</td>
<td>0.15</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>5</td>
<td>0.15</td>
<td>0.01</td>
</tr>
<tr>
<td>6</td>
<td>0.18</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>7</td>
<td>0.34</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>8</td>
<td>0.14</td>
<td>0.05</td>
</tr>
<tr>
<td>9</td>
<td>0.03</td>
<td>0.32</td>
</tr>
</tbody>
</table>

$^1$Experiment conducted in triplicate.
Figure 5.10: Sugar transport and metabolism by Lactococcus lactis.

Adapted from Thompson and Gentry-Weeks (1994). Metabolic end-products other than lactate have been omitted for clarity.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-β-gal</td>
<td>phospho-β-galactosidase</td>
</tr>
<tr>
<td>gal-6P-isom</td>
<td>galactose-6-P isomerase</td>
</tr>
<tr>
<td>tdk</td>
<td>Tagatose-6-phosphate kinase</td>
</tr>
<tr>
<td>aldolase</td>
<td>Tagatose 1,6-bisphosphate aldolase</td>
</tr>
<tr>
<td>D-gal-6P</td>
<td>D-galactose 6P</td>
</tr>
<tr>
<td>D-tag-6P</td>
<td>D-tagatose 6P</td>
</tr>
<tr>
<td>TDP</td>
<td>D-tagatose 1,6 bisphosphate</td>
</tr>
<tr>
<td>DHAP</td>
<td>Dihydroxyacetone phosphate</td>
</tr>
<tr>
<td>pgm</td>
<td>Phosphogluco-mutase</td>
</tr>
<tr>
<td>Galp</td>
<td>galactose permease</td>
</tr>
<tr>
<td>gk</td>
<td>gluco(hexo)kinase</td>
</tr>
<tr>
<td>pgi</td>
<td>phosphoglucone isomerase</td>
</tr>
<tr>
<td>pfk</td>
<td>phosphofructo-kinase</td>
</tr>
<tr>
<td>TDP</td>
<td>Fructose 1,6-bisphosphate aldolase</td>
</tr>
<tr>
<td>GA-3P</td>
<td>Fructose bisphosphate aldolase</td>
</tr>
<tr>
<td>galK</td>
<td>galactokinase</td>
</tr>
<tr>
<td>gal-1P</td>
<td>Galactose 1-phosphate</td>
</tr>
<tr>
<td>G1P</td>
<td>Glucose 1-phosphate</td>
</tr>
<tr>
<td>G6P</td>
<td>Glucose 6-phosphate</td>
</tr>
<tr>
<td>F6P</td>
<td>Fructose 6-phosphate</td>
</tr>
</tbody>
</table>
5.8 Discussion

In a study by Oram and Reiter (1966) with one LPS sensitive and one LPS insensitive strain of *Streptococcus (Lactococcus) lactis*, it was shown that in cell free extracts, the model LPS strongly inhibited hexokinase, the first enzyme in the glycolytic pathway. In this study the objective was to investigate and confirm the effects of LPS inhibition on lactose fermentation by whole cells.

In an initial survey of 13 strains of *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* grown in RSM, a range of sensitivity to the artificial LPS was demonstrated. In contrast to Oram and Reiter's findings (1966), not one of the thirteen commercially used strains was found to be stimulated by the LPS even though LPS mediated inhibition is more effective when H$_2$O$_2$ is generated by an enzyme system rather than from single exogenous additions. From this initial survey, strains exhibiting the extremes in sensitivity were selected for further study.

One factor attributable to variations in LPS sensitivity is the production of H$_2$O$_2$ by the starter culture itself. In the presence of oxygen, the concentration of H$_2$O$_2$ produced by LAB is dependent on the relative activities of the enzymes which form H$_2$O$_2$, for example NADH oxidases, and those which eliminate it, for example NADH peroxidase (Anders et al. 1970). In this study, aeration of the medium was minimised to limit the substrate for H$_2$O$_2$ production and each strain received the same amount of added H$_2$O$_2$. Therefore the ability to eliminate H$_2$O$_2$ could be a variable between the *Lactococcus lactis* strains, which could explain their differences in sensitivity to the model LPS. Although NADH peroxidase is widespread in LAB as a whole, it is not as prevalent among streptococci (Zitzelsberger et al. 1984). Smart and Thomas (1987) reported little or no NADH peroxidase activity in five *Lactococcus lactis* strains grown aerobically or anaerobically and also noted superoxide dismutase (EC 1.15.1.1) activity in all five strains, which catalyses the conversion of
superoxide radicals into molecular oxygen and H$_2$O$_2$. None of the four *L. lactis* strains tested exhibited NADH peroxidase activity. Therefore, among these strains, differences in susceptibility to the LPS cannot be attributed to differences in the ability to eliminate the experimentally added H$_2$O$_2$.

The LPS has been shown to strongly inhibit hexokinase and, to a reduced extent, other glycolytic enzymes of *Lactococcus lactis* (Oram and Reiter 1966). Therefore, the effect of the LPS on the glycolytic enzymes was investigated. Lactococci are able to metabolise D-galactose either via the D-galactose 1-phosphate pathway (Leloir pathway) or via the D-tagatose 6-phosphate pathway (Bissett and Anderson 1974). Thompson and Thomas (1977) observed that starved cells of *Lactococcus lactis* maintained an intracellular PEP concentration and that thiomethyl-β-D-galactopyranoside (a galactose analogue) uptake and PEP utilisation were virtually at a 1:1 stoichiometric ratio. Later, Thomas *et al.* (1980) showed that galactose transport by *L. lactis* subsp. cremoris is predominately via a low affinity phosphotransferase system, the resulting phosphorylated sugar is metabolised via the D-tagatose pathway. Since neither *cremoris* strain 458 nor *cremoris* strain 900 was inhibited when grown in M17 containing galactose, it would appear that this form of galactose transport and the enzymes in further steps of glycolysis, are not inhibited by the LPS. This observation is also compatible with Oram and Reiter’s findings regarding hexokinase inhibition.

No significant inhibition of lactate production by the LPS was evident in *cremoris* strains 458 or 900 when they were grown in M17 containing fructose as the carbon source.

*Lactococcus lactis* transport fructose via the mannose PEP/PTS which is broadly specific for mannose, fructose and glucose, or via an inducible fructose phosphotransferase system, forming either fructose-6-P or fructose 1,6-bisphosphate. This bypasses hexokinase and phosphoglucose isomerase in the glycolytic pathway (Benthin *et al.* 1993). Again, inhibition
by the LPS does not appear to affect either of the fructose transport systems or further steps in glycolysis and also indicates that the inhibition is occurring at some earlier stage in the cell’s metabolism, further supporting Oram and Reiter’s finding.

The fermentation of lactose in M17 broth by both *cremoris* strains 458 and 900 in the presence of the LPS was significantly inhibited. The inhibition of hexokinase could decrease the L-lactate formation when *L. lactis* strains are grown in either RSM or M17 containing lactose as the carbon source, in the presence of the model LPS. Support for Oram and Reiter’s (1966) findings comes from the presumptive hexokinase amino acid sequence for *L. lactis* subsp. *lactis* strain ILI403, which contained five cysteiny1 residues (Bolotin et al. 2001). Cysteine contains a sulfhydryl group (Stryer 1988) which is the oxidation site of the transient LPS products (Thomas and Aune 1978). Further confirmation that hexokinase inhibition is involved in the LPS mediated inhibition of lactose metabolism comes from the observation that a reversal of inhibition occurs in streptococci with the addition of dithiothreitol, which protects sulphhydryl groups (Germaine and Tellefson 1982). In this study, the addition of dithiothreitol into the LPS reaction mixture alleviated the LPS mediated inhibition of *Lactococcus lactis* subsp. *cremoris* strain ASCC 900 when this organism was grown in lactose and glucose.

*Lactococcus lactis* primarily takes up lactose via a PEP/PTS dependent system (McKay et al. 1969) and Thompson (1979) showed that 60% of the intracellular PEP potential was utilised phosphorylating the galactose moiety, producing galactose-6-phosphate, and that an approximately 2:1 molar ratio of PEP utilised to lactose transported, occurred. The remaining glucose moiety is phosphorylated via ATP and hexokinase prior to further metabolism in the glycolytic pathway.

Subsequently, four *Lactococcus lactis* strains were investigated for their ability to utilise glucose in the presence of the model LPS. Strains 307 (*lactis*) and 458 (*cremoris*), both the
least sensitive to the LPS according to the initial survey, were significantly inhibited, as were
the more LPS sensitive strains 441 (*cremoris*) and 900 (*cremoris*). *Lactococcus lactis*
primarily transport glucose via a PEP dependent PTS system, using the multiply specific EII
mannose transport enzyme, which was not inhibited by the LPS when fructose was the
substrate. In this case, glucose is phosphorylated during transport, forming
glucose-6-phosphate which is then metabolised via the glycolytic pathway. Since this form of
glucose metabolism bypasses hexokinase, the LPS inhibition may be due to effects on the
next enzyme in the glycolytic pathway, phosphoglucone isomerase, which Oram and Reiter
(1966) reported as having increased activity in cell free extracts. However, their evidence
indicates the possibility of a secondary glucose transport system in *Lactococcus lactis*.

While there has been relatively little research conducted on glucose transport in
lactococci, more attention has been given to *Streptococcus* spp. associated with human dental
caries and bovine mastitis. A PEP/PTS defective mutant strain of *Streptococcus mutans* was
shown to have a glucose transport system utilising ATP (Cvitkovitch, *et al*. 1995) while a
glucose diffusion mechanism has been demonstrated in *Streptococcus bovis*, a Lancefield
group D streptococcus (Holt 1994, Russell 1990). Although lactococci have an ATP
dependent galactose permease, the system has been shown to have little or no affinity for
glucose or lactose (Thompson 1980). Lawrence *et al*. (1976), commenting on contemporary
research, noted difficulties with claims of glucose phosphorylation via an ATP-linked
mechanism. However, further work by Thompson (1978) demonstrated that in *L. lactis* ML3,
87% of a glucose analogue (2-[^3]H)D-glucose) taken up was found in the phosphorylated
form and 13% existed as the free sugar. Subsequent studies demonstrated glucose transport in
PEP depleted cells, although the capacity of the system appeared limited (Thompson 1980).
The inhibition of L-lactate production by the four *Lactococcus lactis* strains, when grown in
M17 broth with glucose, was somewhat unexpected given that glucose is predominantly
phosphorylated in transit, presumably negating the influence of any LPS mediated inhibition of hexokinase.

It may be that such inhibition could be related to glucose transport via a non-PEP/PTS mechanism. As reported by Thompson (1980), *L. lactis* subsp. *lactis* strain ML3 was able to transport glucose in a starved and PEP depleted state when pre-incubated with arginine, although apparently at a limited rate. Should a sizable proportion of glucose be taken up via non-PEP/PTS mediated transport, the phosphorylation of this free sugar could be dependent on hexokinase. If strains of varying sensitivity were compared, it is possible that the strains more sensitive to the model LPS could be shown to make greater use of this non-PEP mediated glucose transport. Conversely, strains less sensitive to the model LPS may rely more heavily on PEP/PTS mediated glucose transport whereby the majority of the sugar is phosphorylated in transit, reducing the need for hexokinase mediated phosphorylation.

Strains 458 and 900 were assessed for their ability to transport glucose via this system using the reported method (Thompson 1980). In this study, no non-PEP/PTS mediated glucose transport was observed in *L. lactis* subsp. *cremoris* strain 458 or 900.

The ATP dependent transport system of *L. lactis* subsp. *lactis* strain ML3 was less effective at transporting glucose than galactose. Also, transport of the glucose analogue 2-deoxy-D-glucose or lactose via the same pathway was not demonstrated (Thompson 1980). *Lactococcus lactis* subsp. *lactis* mutants lacking the PEP/PTS EII mannose enzyme have been shown to metabolise glucose at only 5% of the rate of their parental strains, while double mutants lacking both the EII mannose enzyme and hexokinase activity were unable to grow on glucose (Thompson and Gentry-Weeks 1994). From these observations, the same authors speculated that *Lactococcus lactis* subsp. *lactis* strains may be able to take up glucose via an “intermediate”, active transport system. However, the inhibition of this minor transport system would not seem to account for the extent of inhibition of L-lactate production by
strains 458 and 900 when grown on glucose in the presence of the LPS, which was reduced by approximately 20 and 60% respectively.

The determination of whether *Lactococcus lactis* subsp. *cremoris* strains even possess this ATP dependent glucose uptake system has been hampered by the inability of the subspecies to metabolise arginine. Thompson (1980) pre-incubated *lactis* strain ML3 with arginine to ensure that the previously starved cells would regenerate sufficient ATP to facilitate glucose transport. *Lactococcus lactis* subsp. *lactis* strains take up arginine via a symport exchange system (Poolman *et al.* 1987). The addition of iodoacetate (IAA) to the culture system prevented the further production of PEP, limiting the system to ATP mediated glucose uptake only. For *Lactococcus lactis* subsp. *cremoris* strains 458 and 900, which by definition do not metabolise arginine (Niven *et al.* 1942), the intracellular ATP regeneration prior to glucose uptake is excluded. Consequently, a method for depleting cells of PEP while not depleting ATP or for regenerating ATP without arginine metabolism, is necessary in order to determine if *Lactococcus lactis* subsp. *cremoris* strains are able to take up glucose via this ATP energised system.

The decrease in L-lactate produced by cells grown in M17 broth with glucose as the carbon source in the presence of the LPS may also indicate that phosphoglucose isomerase is inhibited by the model LPS. Since glucose is predominantly transported via the mannose PEP/PTS where the sugar is phosphorylated in transit, LPS mediated inhibition could occur downstream of hexokinase and based on the observations from fructose metabolism, upstream of phosphofructokinase (EC 2.7.1.11). Inhibition of phosphoglucose isomerase by the LPS could also contribute to the diminished L-lactate production of the cultures grown on lactose. However, there are two points that do not support inhibition of phosphoglucose isomerase by the LPS. The presumptive amino acid sequence for phosphoglucose isomerase in *L. lactis* subsp. *lactis* strain ILI403 contained no cysteinyl residues (Bolotin *et al.* 2001).
while Oram and Reiter (1966) actually observed LPS stimulation of phosphoglucose isomerase activity in cell free extracts.

Finally, a strategy was devised to test for inhibition of phosphoglucose isomerase. Thomas et al. (1980) showed that *L. lactis* subsp. *lactis* makes more use of a high affinity galactose permease system, metabolising the sugar via the Leloir pathway. Using this pathway, the sugar enters the glycolytic pathway as glucose-6-phosphate and consequently is not phosphorylated by hexokinase and ATP.

*Lactococcus lactis* subsp. *lactis* strain ATCC 7962 has been shown to accumulate the galactose analogue thiomethyl-β-galactoside entirely in the free form (Kashket and Wilson (1972). Lawrence et al. (1976) concluded from this finding that the strain lacked a galactose PEP/PTS. Therefore, if *L. lactis* subsp. *lactis* strain ATCC 7962 utilised only an ATP dependent permease system to transport galactose, then hexokinase would be bypassed in the metabolism of this sugar when grown in M17 with galactose as the carbon source. In the present study, the model LPS caused significant inhibition throughout the logarithmic phase of growth of strain ATCC 7962 when grown in M17 containing galactose, although the inhibitory effects were diminished as the culture approached stationary phase. These LPS mediated inhibitory effects cannot be attributed to previously reported inhibition of hexokinase.
6.0 General Discussion

The extent of non-phage inhibition of starter cultures has been examined by this laboratory over two production seasons in a large scale Cheddar manufacturing plant in Victoria, Australia (Mason et al. 2001). The results of this study reinforce the necessity of milk producers and cheese manufacturers having an understanding of the implications of inhibitory factors which may arise from sources remote or unrelated to routine responsibilities. Consequently, this study has investigated technological issues involving antibiotic residue detection, refrigerated milk storage and the response of *Lactococcus lactis* to the native antimicrobial lactoperoxidase system, leading to an investigation of glucose metabolism in the *cremoris* subspecies.

6.1 Limitations of standard antibiotic detection systems

6.1.1 Inadequate sensitivity

Current industry practice for antibiotic screening depends on microbial inhibition assays to provide adequate broad spectrum detection sensitivity. Besides antibiotic sensitivity, these test methods are chosen because they are rapid, have relatively low setup and maintenance costs, require little specialised hardware or technical training and the results are available within reasonable time periods. The assumption however, that standard antibiotic screening tests are sufficiently sensitive appears unfounded in light of the data reported here. Previous research into establishing the causes of non-phage inhibition, may also have to be reevaluated. For example, Roginski et al. (1984a) who surveyed Victorian milks over one
production season to determine the extent of non-phage inhibition, relied on the standard BSDA to eliminate antibiotic residues as causal agents. In this case it is possible that some of the inhibitory milks assessed may have contained sufficiently high antibiotic concentrations to inhibit starter cultures.

Generally, it may be assumed that any antibiotic residues occurring in milk become diluted during the transport and silo storage phases of large scale commercial processes. However, this assumption does not take into account occasions where tankers may not be completely filled and/or where a partially full silo may be topped up (Mason et al. 2001).

6.1.2 Risk assessment of undetected antibiotic residues

Mason et al. (2001) determined the minimum inhibitory concentrations of a selection of antibiotics approved for use on lactating cattle against a range of Lactococcus lactis starter strains. The survey revealed that on average, cremoris strains were inhibited at concentrations 65% lower than lactis strains and that inhibition varied with the type of antibiotic. The strains tested were least sensitive to neomycin and as this study also showed, this antibiotic was readily detected by the Delvo SP, indicating the minimal risk to cheese manufactures of neomycin residues in milk.

In contrast, Mason et al. (2001) showed that the minimum inhibitory concentration of novobiocin was the lowest in the antibiotic range tested against Lactococcus lactis strains surveyed. Consequently, as a result of the widespread reliance on the Delvo SP and its insufficient sensitivity to novobiocin, the greater sensitivity of starter cultures to the antibiotic, and its increasing use, risk of inexplicable starter culture inhibition through the use of this antibiotic is high (Mason et al. 2001).
6.1.3 Specific recommendations

In light of the present study’s data, a recommendation could be made that processors develop a strategy to employ rapid, highly sensitive antibiotic specific assays targeting non-β-lactam antibiotics. Responsible parties could base decisions to stock such assays on survey results referred to in this paper and historical data collected on-site.

Further, strategies for management of starter cultures should incorporate culture screening to identify starter strains that are less inhibited at low antibiotic concentrations. Orberg and Sandine (1985) reported variability in resistance among lactic streptococci to lincomycin and neomycin, although they concluded that specific antibiotic resistance is rare in these organisms. Valladao and Sandine (1994) recommended a disk assay method to survey antibiotic resistance among starter cultures and identified a range of sensitivity to various antibiotics. They also suggested the use of the method as an antibiotic screening assay.

In the long term however, reducing the need for intramammary antibiotics would be the most desirable outcome. Reducing the incidence of mastitis in the national herd benefits the producer, processor and consumer alike. Programmes such as Countdown Downunder implemented in Australia, are aimed at reducing somatic cell counts, the primary indicator of udder health.

6.2 Acid production by a L. lactis in temperature abused milk

The second part of this study explored the likelihood of adverse effects on starter culture acid production in milk stored for prolonged periods at elevated temperatures leading to elevated levels of lipolytic and proteolytic degradation products. It is known that psychrotrophic bacteria have the potential to produce heat resistant lipase which can lead to lipolytic degradation products inhibitory to some lactic acid bacteria. However, no evidence of starter culture inhibition was found under the experimental conditions. Further, for most
large-scale operations, the existing threats to cold chain maintenance are well understood. Bulk tank temperature monitoring, vigilance in silo turnover and other quality assurance procedures should not only ensure the prevention of starter difficulties, but also a number of other well described quality defects attributable to milk fat degradation prior to processing.

6.3 Sensitivity of *L. lactis* strains to a model LPS

In this section of the study the inhibitory effect of natural antimicrobial systems in bovine milk on starter cultures used in cheese manufacture was investigated. Since *Lactococcus lactis* subsp. *cremoris* and subsp. *lactis* strains are used worldwide in Cheddar cheese manufacture, the dairy industry would benefit from an enhanced understanding of their sensitivity to native antimicrobial systems. This study has shown that the growth and acid production of most lactococci are affected by the lactoperoxidase system, leaving some questions as yet unanswered from the research conducted previously. No link between the decrease of lactate production with glucose as the carbon source and inhibition of hexokinase by the LPS is apparent. In addition, with lactose as the carbon source, the decrease in L-lactate production may not conclusively be explained by the inhibition of hexokinase as unphosphorylated PTS substrates have been shown to be phosphorylated intracellularly by the PTS (Thompson and Chassey 1985). Also, a better understanding is required of the non-PEP/PTS dependent transport of glucose by PEP depleted cells of *Lactococcus lactis* subsp. *lactis* and the failure to transport the glucose analogue 2-deoxy-D-glucose by the same system (Thompson 1980). Further investigations to determine whether non-PEP/PTS dependent sugar transport systems are available to *cremoris* strains are desirable. With the publication of the *Lactococcus* genome (Bolotin *et al.* 2001), molecular explanations for non-PEP/PTS glucose transport mechanisms may be forthcoming. Strain managers could consider adding LPS sensitivity information to their list of characteristics, given the reports of
LPS stimulated lactococcal strains (Oram and Reiter 1966) and the variability in sensitivity as
demonstrated in this study.

6.4 Investigations into non-phage inhibition

Investigations into non-phage inhibition by this laboratory have been hampered by the
difficulty in obtaining inhibitory milk samples for analysis (Mason, pers. comm.).
Commercial logistics prohibit holding samples of every vat until makesheets are examined.
Further, recognising an inhibited vat early enough to collect a milk or whey sample for
analysis is beyond the scope of typical responsibilities of factory personell (Limsowtin, pers.
comm.)

6.5 Quality assurance recommendations

The present study has explored issues of milk quality, the consequences of which lend
support to recommendations previously made by this laboratory (Mason et al. 2001). Among
the recommendations are that:

i. milk storage silos be completely emptied before refilling and be refilled to capacity.
ii. hydrogen peroxide residue risks be assessed and reduced where possible with respect
to cheese making vats.

Adherence to these two points would increase the chances of diluting any potential
inhibitors, minimise the storage time of all incoming milk and reduce the likelihood of
activating the native antimicrobial LPS. From a quality control perspective, two further
recommendations may be considered:

i. specifically targeted antibiotic screening assays be employed when testing farm vat or
individual cow milk samples.

ii. Starter Addition Tests (SAT) be developed for silo milk testing prior to use.
The technology to fulfill the first recommendation certainly exists. Research must continue towards developing the second. Management, seeking the elimination of non-phage inhibition difficulties in large scale Cheddar cheese manufacture, would be well advised to include these recommendations in their overall QC approaches.


References


References


Fairbairn, D.J. and Law, B.A. (1986), Proteinases of psychrotrophic bacteria: their production, properties, effects and control. J. Dairy Res. 53, 139-177.


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Appendices

A1

Table A1: List of *L. lactis* strains

<table>
<thead>
<tr>
<th>ASCC strain number</th>
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<tr>
<td>47</td>
<td>cremoris</td>
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<tr>
<td>92</td>
<td>cremoris</td>
</tr>
<tr>
<td>249&lt;sup&gt;2&lt;/sup&gt;</td>
<td>lactis</td>
</tr>
<tr>
<td>307</td>
<td>lactis</td>
</tr>
<tr>
<td>441</td>
<td>cremoris</td>
</tr>
<tr>
<td>458</td>
<td>cremoris</td>
</tr>
<tr>
<td>519</td>
<td>lactis</td>
</tr>
<tr>
<td>531</td>
<td>cremoris</td>
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<tr>
<td>651</td>
<td>lactis</td>
</tr>
<tr>
<td>818</td>
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</tr>
<tr>
<td>900</td>
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<tr>
<td>995</td>
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<tr>
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<tr>
<td>1080</td>
<td>lactis</td>
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<tr>
<td>ATCC 7962</td>
<td>lactis</td>
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</table>

<sup>1</sup> Determined by arginine metabolism

<sup>2</sup> Referred to in the literature as ML3.
Figure A2: Determination of late log phase for *L. lactis* subsp. *cremoris* cultures

pH measurements of the strains listed incubated in RSM as described in Materials and Methods.
Figure A3: Determination of late log phase for *L. lactis* subsp. *lactis* cultures

pH measurements of the strains listed incubated in RSM as described in Materials and Methods.
Figure A4: Typical growth of *L. lactis* strains 458 and 900 in M17 lactose.

Growth of *L. lactis* subsp. *cremoris* strains ASCC 458 and 900 in M17 lactose. Values are means of triplicate experiments determined by measuring the OD @ 600nm as described in Materials and Methods.
Figure A5: Typical growth of *L. lactis* strains 458 and 900 in M17 glucose.

Growth of *L. lactis* subsp. *cremoris* strains ASCC 458 and 900 in M17 glucose. Values are means of triplicate experiments determined by measuring the OD @ 600nm as described in Materials and Methods.
Figure A6: Typical growth of *L. lactis* strains 458 and 900 in M17 galactose.

Growth of *L. lactis* subsp. *cremoris* strains ASCC 458 and 900 in M17 galactose. Values are means of triplicate experiments determined by measuring the OD @ 600nm as described in Materials and Methods.
Figure A7: Typical growth of *L. lactis* strains 458 and 900 in M17 fructose.

Growth of *L. lactis* subsp. *cremoris* strains ASCC 458 and 900 in M17 fructose. Values are means of triplicate experiments determined by measuring the OD @ 600nm as described in Materials and Methods.
### Table A8: Relationship of optical density to cell dry weight

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<tr>
<td>307</td>
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<tr>
<td>441</td>
<td>0.0035</td>
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<tr>
<td>458</td>
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</tr>
<tr>
<td>900</td>
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<td>ATCC 7962</td>
<td>0.0041</td>
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¹ Slope calculated from 5 or 6 points. All R² values >0.96.
² ASCC numbers except ATCC 7962
### Table A9: List of media, chemicals and reagents

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<td>bovine serum albumin</td>
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<td>Sigma</td>
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<td>cloxacin</td>
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<td>D-[U-14C]-glucose</td>
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<td>2-deoxy-D-glucose</td>
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### Table A10: List of equipment

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<td>antibiotic detection</td>
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<td>chart recorder</td>
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<tr>
<td>sonicator</td>
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Non-Phage Inhibition of Cheese Starter Lactococci

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<th>Device</th>
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<td>thermometer: digital</td>
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Table A11: Antibiotic formulations approved for use on lactating dairy cattle

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<td>Ampiclox L.C. lactating cow intramammary antibiotic infusion</td>
<td>Jurox Pty Ltd</td>
<td>500.00 mg/Sg, 75,000 mg/Sg, 2000.000 mg/Sg</td>
<td>Cloxacillin Benzathine, Ampicillin as the sodium salt, Cloxacillin as the sodium salt</td>
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<td>38,696</td>
<td>Special formula 17900 forte-v lactating intramammary antibiotic suspension</td>
<td>Pharmacia &amp; Up John Pty Ltd</td>
<td>100.000 mg/10ml, 100.000 mg/10ml, 150.000 mg/10ml</td>
<td>Dihydrostreptomycin, Novobiocin as Novobiocin sodium, Neomycin sulfate</td>
</tr>
<tr>
<td>38,698</td>
<td>Lincocin forte lactating intramammary antibiotic solution</td>
<td>Pharmacia &amp; Up John Pty Ltd</td>
<td>200,000 mg/ml, 200,000 mg/ml</td>
<td>Lincomycin as Lincomycin Hydrochloride, Neomycin</td>
</tr>
<tr>
<td>46,337</td>
<td>Orbenin L.C. lactating cow intramammary antibiotic infusion with prolonged action</td>
<td>Pfizer Animal Health a div of Pfizer Pty Ltd</td>
<td>200,000 mg/Sg</td>
<td>Cloxacillin as the benzathine salt</td>
</tr>
<tr>
<td>46,904</td>
<td>Mastop suspension for the treatment of mastitis in lactating cows</td>
<td>Pharmacia &amp; Up John Pty Ltd</td>
<td>150,000 mg/Pt, 100,000,000 IU/Pt</td>
<td>Novobiocin as Novobiocin Sodium Procaine Penicillin</td>
</tr>
<tr>
<td>47,941</td>
<td>Coopers Cepravin L.C. lactating cow intramammary antibiotic</td>
<td>Schering-Plough Animal Health Ltd</td>
<td>92,200 g/kg</td>
<td>Cefuroxime Sodium</td>
</tr>
<tr>
<td>49,851</td>
<td>Mastalone blue intramammary suspension for lactating cows</td>
<td>Pfizer Animal Health A Division of Pfizer Pty Ltd</td>
<td>100,000 mg/10ml, 100,000 mg/10ml, 185,000 mg/10ml</td>
<td>Neomycin, Oleandomycin, Oxytetracycline as Oxytetracycline Hydrochloride</td>
</tr>
</tbody>
</table>

National Registration Authority 1999
# A12

## Table A12: Antibiotic chemical and drug family

<table>
<thead>
<tr>
<th>Antibiotic chemical</th>
<th>Drug family</th>
</tr>
</thead>
<tbody>
<tr>
<td>ampicillin</td>
<td>β-lactam</td>
</tr>
<tr>
<td>cloxacillin</td>
<td>β-lactam</td>
</tr>
<tr>
<td>penicillin</td>
<td>β-lactam</td>
</tr>
<tr>
<td>cefuroxime</td>
<td>β-lactam/cephalosporin</td>
</tr>
<tr>
<td>lincomycin</td>
<td>macrolide</td>
</tr>
<tr>
<td>oleandomycin</td>
<td>macrolide</td>
</tr>
<tr>
<td>neomycin</td>
<td>aminoglycoside</td>
</tr>
<tr>
<td>dihydrostreptomycin</td>
<td>aminoglycoside</td>
</tr>
<tr>
<td>novobiocin</td>
<td>unclassified</td>
</tr>
</tbody>
</table>
Abstract:

A range of antibiotics registered for therapeutic use on lactating cattle was assessed for their inhibitory effect on starter lactic acid bacteria. A number of non-β-lactam antibiotics, novobiocin, lincomycin, oleandomycin and oxytetracyline HCl inhibited acid production of one or more *L. lactis* strains grown in reconstituted skim milk. The inhibitory concentrations of the antibiotics were in some instances below their detectable limits using either or both the *Bacillus stearothermophilus* (var. *calidolactis*) disk assay and the Delvo SP assay. The presence of specific antibiotic residues in milk may inhibit acid production by starter bacteria and significantly affect the cheese making process leading to longer make times and the disruption of cheese making schedules.

Excerpt from project summary:

The most commonly used of the antibiotics, according to the survey, were assessed against a series of Cheddar starter cultures in order to determine the effect of antibiotics at concentrations approaching the lower detection limits of the Delvotest SP antibiotic test system which is used throughout the Australian dairy industry. It was evident that novobiocin, oxytetracyline, oleandomycin and lincomycin at levels below that able to be detected by the Delvotest SP method, significantly inhibited starter culture activity in milk. Interestingly, two out of the three principal commercial intramammary antibiotic formulations used in the survey region contained these antibiotics.

Poster presentation:

A16

Seminar presentation¹:


¹ Data from present study included in presentation.
Author/s:
Packham, Wayne

Title:
Non-phage inhibition of cheese starter lactococci

Date:
2002

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