STUDIES ON THE PREVALENCE, PERSISTENCE AND ANTIBIOTIC RESISTANCE OF ENTEROCOCCI FROM AUSTRALIAN DAIRY SOURCES

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This thesis is being submitted in total fulfilment of the degree

of

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Faculty of Veterinary and Agricultural Sciences

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ABSTRACT

This work was undertaken to determine the incidence of enterococci in raw milk and dairy products in the dairying regions of south-eastern Australia over a one-year period and to assess the ability of enterococci to persist in the dairy environment, survive pasteurisation and grow in dairy products. In addition, the risks associated with the presence of enterococci in dairy products was also considered.

Enterococci were widespread in milk prior to manufacture, with lower counts occurring in raw milk in winter, which has not been reported in other studies internationally. The level of detection of enterococci in pasteurised milk was low and did not coincide with higher raw milk counts, indicating that the main enterococci population was heat sensitive. Although Enterococcus faecalis was the predominant species found in the raw milk, E. faecalis, E. malodoratus and E. faecium were detected in the greatest number of butter, Cheddar cheese and milk powder samples, respectively, suggesting that these species have inherent properties that enable them to survive processing conditions used in the manufacture of these dairy products.

Thermal death determinations of the four main species identified from laboratory pasteurised milk (63 °C, 30 min) revealed that the E. faecalis isolates were the most heat sensitive, with the lowest z values (5.0-7.5 °C), while E. faecium, E. hirae and E. durans had the highest z values (8.5-9.8 °C). Thermoduric enterococci were able to grow at 4 and 7 °C in UHT milk. They were able to survive but not grow in butter, Cheddar cheese, cottage cheese, milk powder and yoghurt and in acidic and higher salt concentrations at 4 °C, indicating that dairy product environments limit the growth of enterococci.

Genetic fingerprinting of enterococci at one of the dairy factories identified a persistent population of E. faecalis, which had streptomycin resistance and increased biofilm production, and inferred a link between these traits and persistence.
The most frequently encountered antibiotic resistances were to streptomycin and tetracycline, which, along with chloramphenicol and erythromycin resistance, could be transferred to both *E. faecalis* and *E. faecium* but in limited situations to *E. hirae* and *Listeria monocytogenes*. Transfer to *E. hirae* has not been previously demonstrated, which is significant in context of this species increased resistance to heat treatment. The isolates were also mated in a multi-strain biofilm on stainless steel coupons in milk incubated at 10 and 25 °C. No transconjugants were detected under these experimental conditions.

Results of this study found that enterococci in the south-eastern Australian raw milk supply possessed multiple virulence factors and antibiotic resistances, which were at times transferrable between species and genera. While the majority of enterococci were eliminated with pasteurisation, some enterococci were heat-resistant, meaning that enterococci with virulence factors and antibiotic resistance could gain entry into the food supply if these traits were present in such isolates. However, resistance to the antibiotic of most concern, vancomycin, was not found in *E. faecalis*, a major species associated with medical issues. Although there is a theoretical risk of enterococci containing medically-undesirable traits entering the food supply, their ability to grow in products may be limited by the nature and storage conditions of the products.
DECLARATION

- This thesis comprises only my original work towards the Doctor of Philosophy except where indicated in the preface,
- due acknowledgement has been made in the text to all other material used,
- the thesis is fewer than the maximum word limit in length, exclusive of tables, maps, bibliographies and appendices.

Catherine McAuley
21 July 2016
The research conducted for this thesis was performed entirely at the research institution located at 671 Sneydes Rd., Werribee, Victoria. From 2002 to 30 May, 2004, this institution was known as Food Science Australia, which was a joint venture of the Australian Food Industry Science Centre and the Commonwealth Scientific and Industrial Research Organisation (CSIRO). After this time, the institution became wholly CSIRO. This PhD commenced in 2002 and was full-time for the first year. Thereafter, the research was conducted part-time, in conjunction with work. During the period of lapsed candidature, I was working full time and gave birth to my son.

The following publications were submitted during the course of my PhD candidature:


The following work is in preparation for publication:


In association with Dr Heather Craven, I conceived the ideas for all publications. I planned, conducted and analysed all of the experimental work, prepared the draft of all of the publications and responded to comments from journal reviewers. Drs Heather Craven and Kari Gobius, and Prof Margaret Britz contributed to the editing of the draft
publications. Dr Heather Craven and Prof Margaret Britz contributed to editing in response to reviewers comments. The co-author authorisation forms and the declaration for thesis with publication forms for publications 1 and 2 are submitted as part of this thesis submission.

Chapter 2 is prepared as a traditional thesis chapter incorporating the presence and seasonality of enterococci published in publication 1 (Appendix A), with an expanded methods section and additional work. The survey of raw milk and dairy products was statistically designed by Dr John Reynolds and statistically analysed in part by Dr Murray Hannah, Principle Biometricians at the Department of Primary Industries, Victoria.

Chapter 3 is based on publication 2 (Appendix B), with an expanded methods section.

Chapter 4 is prepared as a traditional thesis chapter and incorporates the growth of enterococci in raw and pasteurised milk, which was published in publication 1.

Chapter 5 is being prepared as a publication (publication 3).

Chapters 6 and 7 are traditional thesis chapters. Although the virulence work in Chapter 6 was conceived and analysed by me, the laboratory testing was conducted by others. Dr Kerryn King at Food Science Australia performed the PCR for the *agg* and *esp* genes. Judith Vaessen, a Masters student from Wageningen, The Netherlands, conducted the gelatinase and haemolysis tests, under my supervision in the laboratory.

Funding for the work was provided in part at different times by Dairy Australia, the Geoffrey Gardiner Dairy Foundation Grant MP1/016, the Department of Natural Resources and Environment (later the Department of Primary Industries, Victoria and presently the Department of Economic Development, Jobs, Transport and Resources) and CSIRO.
ACKNOWLEDGEMENTS

First of all, I would like to thank my long-running supervisors Drs Heather Craven and Kari Gobius and Professor Margaret Britz for all of their help and unwavering support over the years and not giving up on me! Their dedication, flexibility and help in the final weeks were outstanding. Heather guided the project from its conception and was my daily support and an excellent teacher. Heather’s knowledge of the dairy industry was invaluable. I very much appreciated Heather’s steadfast presence in the final days, spending late nights and days off to see this through. Kari’s assistance in navigating the CSIRO publishing requirements was much appreciated. Margaret’s amazing breadth and depth of knowledge throughout the project assisted with the quality of the work. Regardless of work commitments, holidays and being Dean, Margaret was always quick to respond to my queries and comment on documents, and I am sure is leagues ahead of most university supervisors.

I would like to thank Dr Kerryn King for so many things. Kerryn was a continuing support in the lab throughout all of my experiments and has always been a good friend. I am particularly thankful for her help with last minute queries in the final weeks prior to submission.

The staff and students at Food Science Australia and then CSIRO have provided continued assistance and moral support, particularly in the final weeks when people would stop me in the hall and offer encouragement. Many thanks to Dr Frank Wong for getting me started in the world of molecular microbiology and making PCR and PFGE less frightening. I especially need to thank Mrs Marie Collier for making the enormous volumes of microbiological media that were required for this work. Sieh Ng’s friendship and cheery outlook helped ease the stress when things were getting tense. I would like to thank Drs John Coventry and Jocelyn Midgley for their helpful comments in reviewing the Dairy Australia report, which contributed to chapters 2-4 and 6.
I gratefully acknowledge the expert advice of Drs John Reynolds and Murray Hannah, Principle Biometricians, Department of Primary Industries, Victoria for the statistical design and the analysis of results of the survey to determine the prevalence of enterococci in the Australian dairy industry.

Projects like this could not proceed without the kind participation of the dairy factories that made this work possible. Of course they would not like to be named but their support in providing samples, monthly for an entire year in some cases, is much appreciated.

Finally, of course, I would like to thank my family, my parents, Peter and Margaret McAuley, and my husband, Geoff Knight, for their love and support. Geoff accompanied me in the lab through the long hours that were the dairy survey, with only a day off a month! Geoff has always been there for me and I would not have been able to finish my thesis without his help in holding the fort at home. My seven year old son Aidan probably wonders why his Mom always had to “go to work” on the weekends. He will now get his Mom back and maybe one day will understand what it was all about!
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2XCEB</td>
<td>Double strength Chromocult Enterococci Broth</td>
</tr>
<tr>
<td>Ace</td>
<td>Adhesin of collagen</td>
</tr>
<tr>
<td>ADH</td>
<td>Arginine Dehydrolase</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>AS</td>
<td>Aggregation Substance</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BCCM</td>
<td>Belgian Coordinated Collections of Microorganisms</td>
</tr>
<tr>
<td>BHIA</td>
<td>Brain Heart Infusion Agar</td>
</tr>
<tr>
<td>BHIB</td>
<td>Brain Heart Infusion Broth</td>
</tr>
<tr>
<td>BEA</td>
<td>Bile Esulin Agar</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CEB</td>
<td>Chromocult Enterococci Broth</td>
</tr>
<tr>
<td>CEBA</td>
<td>Chromocult Enterococci Broth with Agar</td>
</tr>
<tr>
<td>Chlor</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>Cip</td>
<td>Ciprofloxacin</td>
</tr>
<tr>
<td>CSIRO</td>
<td>Commonwealth Scientific and Industrial Research Organisation</td>
</tr>
<tr>
<td>ECA</td>
<td>Enterococcosel Agar</td>
</tr>
<tr>
<td>Ery</td>
<td>Erythromycin</td>
</tr>
<tr>
<td>ESP</td>
<td>Enterococcal Surface Protein</td>
</tr>
<tr>
<td>FSA</td>
<td>Food Science Australia</td>
</tr>
<tr>
<td>Gen</td>
<td>Gentamicin</td>
</tr>
<tr>
<td>KF Strep</td>
<td>KF Streptococcus Agar</td>
</tr>
<tr>
<td>LAB</td>
<td>Lactic Acid Bacteria</td>
</tr>
<tr>
<td>LAP</td>
<td>Leucine Aminopeptidase</td>
</tr>
<tr>
<td>Mac2</td>
<td>MacConkey Agar No. 2</td>
</tr>
<tr>
<td>MHA</td>
<td>Mueller Hinton Agar</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
</tr>
<tr>
<td>MIC90</td>
<td>Minimum Inhibitory Concentration of 90 % of the population</td>
</tr>
<tr>
<td>MSCRAMM</td>
<td>Microbial surface components recognising adhesive matrix molecules</td>
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<tr>
<td>NA</td>
<td>Nutrient Agar</td>
</tr>
<tr>
<td>xx</td>
<td>xx</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>NB</td>
<td>Nutrient Broth</td>
</tr>
<tr>
<td>NCCLS</td>
<td>National Committee for Clinical Laboratory Standards</td>
</tr>
<tr>
<td>Nit</td>
<td>Nitrofurantoin</td>
</tr>
<tr>
<td>NSLAB</td>
<td>Non-starter Lactic Acid Bacteria</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Pen</td>
<td>Penicillin</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulse Field Gel Electrophoresis</td>
</tr>
<tr>
<td>PYR</td>
<td>Pyrrolidonyl Aminopeptidase</td>
</tr>
<tr>
<td>Rif</td>
<td>Rifampin</td>
</tr>
<tr>
<td>SGPP</td>
<td>Sodium glycerophosphate</td>
</tr>
<tr>
<td>SBA</td>
<td>Slanetz and Bartley Medium (Agar)</td>
</tr>
<tr>
<td>Strep</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>Tet</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptone Soya Broth</td>
</tr>
<tr>
<td>TSYEA</td>
<td>Tryptone Soya Yeast Extract Agar</td>
</tr>
<tr>
<td>TTC</td>
<td>Triphenyl tetrazolium chloride</td>
</tr>
<tr>
<td>Van</td>
<td>Vancomycin</td>
</tr>
<tr>
<td>VCEBA</td>
<td>Vancomycin Chromocult Enterococci Broth with Agar</td>
</tr>
<tr>
<td>VRE</td>
<td>Vancomycin-resistant enterococci</td>
</tr>
<tr>
<td>X-GLU</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside</td>
</tr>
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CHAPTER 1 - GENERAL INTRODUCTION
1.1 The genus *Enterococcus*

The taxonomy of the genus *Enterococcus* has had a long and varied history which was documented by Devries and Pot in 1995. Enterococci were first described by Thiercelin in 1899, which Thiercelin had termed the Gram-positive diplococcus “entérocoque” (Devriese and Pot, 1995). Not long after, the species was renamed *Streptococcus faecalis* by Andrewes and Horder in 1906 due to the observed capability of forming short or long chains (Devriese and Pot, 1995). Streptococci were classified serologically by Rebecca Lancefield in 1933 (Lancefield, 1933). At that time, the enterococci belonged to the Lancefield Group D streptococci (Devriese and Pot, 1995). Interestingly, all of the original isolates that Lancefield had designated Group D were obtained from cheese and were not considered to cause human disease (Lancefield, 1933). In 1937, Sherman divided the streptococci into four groups: enterococci, lactic streptococci, viridans streptococci and pyogenic streptococci, and in 1978, Jones re-termed the enterococci as faecal streptococci (Devriese and Pot, 1995), which is a term that occasionally still arises. The name *Streptococcus faecalis* persisted until 1984 when it, along with *Streptococcus faecium*, was transferred to the revived genus *Enterococcus* (Schleifer and Kilpper-Bälz, 1984).

The genus *Enterococcus* is catalase-negative, Gram-positive cocci with a number of physiological traits that are used to differentiate them from other genera (Facklam et al., 2002). These phenotypic characteristics include the ability to: hydrolyse esculin in the presence of 40 % bile salts, grow at 10 and 45 °C, grow in 6.5 % NaCl, produce leucine aminopeptidase (LAP) and hydrolyse pyrrolidonyl-β-naphthylamide (Facklam et al., 2002). Yellow pigmentation is present in only limited species, including *E. casseliflavus*, *E. sulfureus* and *E. mundtii* (Lebreton et al., 2014). The genera have a DNA G + C content ranging from 37-45 mol % with a genome ranging from 2.7-3.6 Mb (Lebreton et al., 2014). At the commencement of the work presented here, there were 19 described species of enterococci (Devriese and Pot, 1995). Presently, in July 2016, there are at least 50 species (Table 1.1).
Table 1.1 Current species (2016) of enterococci presented in their species groups based on 16S rRNA gene similarity

<table>
<thead>
<tr>
<th>Enterococcus species group</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. avium group</strong></td>
<td>E. avium, E. devriesei, E. gilvus, E. malodoratus, E. pseudoavium, E. raffinosus, <strong>E. viikkiensis</strong>, <strong>E. xiangfangensis</strong></td>
</tr>
<tr>
<td><strong>E. cecorum group</strong></td>
<td>E. cecorum, E columbiae</td>
</tr>
<tr>
<td><strong>E. dispar group</strong></td>
<td>E. dispar, E. asini, E. canintestini, E. hermanniensis, E. pallens, <strong>E. diestrammenae</strong></td>
</tr>
<tr>
<td><strong>E. faecium group</strong></td>
<td>E. faecium, E. canis, E. durans, E. hirae, E. mundtii, E. phoeniculicola, E. ratti, E. villorum, E. thailandicus, <strong>E. lactis</strong></td>
</tr>
<tr>
<td><strong>E. gallinarum group</strong></td>
<td>E. gallinarum, E. casseliflavus</td>
</tr>
<tr>
<td><strong>E. saccharolyticus group</strong></td>
<td>E. saccharolyticus, E. acquimarinus, E. camelliae, E. italicus, E. sulfureus, <strong>E. lemanii, E. eurekensis</strong>, <strong>E. alcedinis, E. olivae, E. bulliens</strong></td>
</tr>
</tbody>
</table>

Table is taken from Franz et al. (2011). Additional species are indicated in bold obtained from information in the publications by Rahkila et al. (2011); Morandi et al. (2012); Niemi et al. (2012); Švec et al. (2012); Cotta et al. (2013); Frolková et al. (2013); Kim et al. (2013); Sedláček et al. (2013); Li et al. (2014); Lucena-Padrós et al. (2014); Kadri et al. (2015).
1.2 Habitats

The word ubiquitous is often used to describe the habitat of enterococci due to their widespread occurrence in the environment. They have been found in wild birds (Roberts et al., 2015), domestic fowl (Bertelloni et al., 2015), beaches and children’s sandboxes (Staley et al., 2016), environmental freshwater (Veljović et al., 2015), seawater (Brown and Boehm, 2015), a range of small animals (rats, voles, mice, squirrel, shrew) (Lozano et al., 2015), fish (Araújo et al., 2015), soil (Bucci et al., 2015), house flies (Doud et al., 2014), domestic pets (cats and dogs) (Roderigues et al., 2002), plants (Ott et al., 2001; Švec et al., 2012) and various farm animals, including cows (Devriese et al., 1992), goats (Cortés et al., 2006), pigs (Jackson et al., 2005), horses (Lauková et al., 2008) and sheep (Moriarty et al., 2011). Enterococci are intestinal microorganisms (Franz et al., 1999) and many of the occurrences listed here are either faecal in origin or can be contaminated by faeces. In addition to wastewater treatment plants, enterococci have been isolated from river water and storm water in Australia, where they were considered to be indicators of faecal contamination (Rathnayake et al., 2012; Sidhu et al., 2012; Ahmed et al., 2016). This is particularly relevant for the farm environment, from which the food supply originates. The following sections describe the habitats of enterococci.

1.2.1 Farm and environment

Enterococci have been investigated on dairy farms in the US (Jackson et al., 2011) but not presently investigated in Australia, as far as what has been published. The most prevalent enterococci species on the US dairy farms were *E. hirae*, *E. faecium* and then *E. faecalis*, with the highest proportion of antibiotic resistances being to linomycin, flavomycin and tetracycline (Jackson et al., 2011). A study which was designed to compare the recovery of enterococci in feedlot cattle manure from five feedlots from Victoria to Queensland, Australia, using quantitative real-time PCR and cultural...
techniques incidentally found that the majority of the enterococci were *E. faecalis* (Klein et al., 2010).

Enterococci are common inhabitants of mammalian intestines (Stiles, 1989), although this can vary with the age of the animal. A range of enterococci species have been isolated from pre-ruminating calves whereas the detection of enterococci in the feces of dairy cows was rare (Devriese et al., 1992). Non-human cases of enterococcal infections are periodically reported in chickens due to *E. cecorum*, *E. durans* and *E. hirae* (for example, see Chadfield et al., 2005; Abe et al., 2008; Jung and Rautenschlein, 2014), although *E. faecalis* has also caused infections (Petersen et al., 2008). Enterococci can cause bovine mastitis (Ganda et al., 2016) but are not reported often for causing other disease in animals. However, they have been investigated in animals for the presence of antibiotic resistance (Jackson et al., 2010; Kojima et al., 2010; Fard et al., 2011; Hamilton et al., 2013; Beukers et al., 2015).

In some parts of the world, the use of antibiotics as growth promotants has led to the development of antibiotic resistance in enterococci in animals (Bonten et al., 2001; Beukers et al., 2015). Cross-resistance to vancomycin, erythromycin and quinupristin/dalfopristin has developed through the use of avoparcin, tylosin and virginiamycin in farm animals (Hammerum et al., 2010; Beukers et al., 2015). The concern is that enterococci present in food may have acquired antibiotic resistance as a result the agricultural use of antibiotics and transfer this to other lactic acid bacteria (Franz et al., 1999) or to more pathogenic Gram-positive bacteria (Giraffa, 2002). This led to a ban on avoparcin and virginiamycin in Europe (Hammerum et al., 2010). An influential paper by Bonten et al. (2001) in *The Lancet* particularly brought the issue to the forefront. After the ban of avoparcin and virginiamycin, the incidence of antibiotic resistance has decreased, although antibiotic resistance in enterococci has persisted in animals and the environment in some instances (Bonten et al., 2001; Hammerum et al., 2010). In the US, a trial in which tylosin use was discontinued in beef cattle found that there was a decrease in enterococci resistant to erythromycin after the discontinuation (Beukers et al., 2015). In Australia, avoparcin was withdrawn from use in farm animals in 2000, and a survey of enterococci four years later found no resistance to this
antibiotic or acquired vancomycin resistance in pigs, although the incidence of these resistances prior to the ban was unknown (Barton et al., 2003; Hart et al., 2004). In poultry, a low number of vancomycin-resistant enterococci were detected before the avoparcin ban, but were not detected a decade after the ban (Barton et al., 2003; Obeng et al., 2013). It seems clear that although there may not be an immediate decrease in antibiotic resistance in enterococci, the discontinuation of agricultural antibiotics that may confer resistance to medically important antibiotics was justified and should be maintained if a mitigation of antibiotic resistance in food animals is desired (Hammerum et al., 2007).

1.2.2 Clinical
Nosocomial infections from enterococci have increased since the 1970’s (Murray, 1990a). The combination of antibiotic resistance and virulence properties of enterococci have contributed to the success of enterococci as pathogens. Clinical infections due to enterococci are varied and include bacteraemia, urinary tract infections, intra-abdominal and pelvic infections, tissue and wound infections, neonatal infections, cholecystitis, meningitis and endocarditis, which has been known for over 100 years (Kayser, 2003; Arias and Murray, 2012; Rubinstein and Keynan, 2013). While some of the infections are community acquired, others are nosocomial (Arias and Murray, 2012). In particular, the risk factors associated with nosocomial infections include prolonged hospital stays, organ transplants, urinary catheters, proximity to other patients with vancomycin-resistant enterococci (VRE), care in surgical or intensive care units, multiple antibiotic therapies and serious concurrent underlying illnesses (Kayser, 2003; Arias and Murray, 2012).

1.2.3 Food
The presence of enterococci in food has been investigated for a long time (Batish and Ranganathan, 1984a; Rao et al., 1986; Wessels et al., 1988) and in subsequent years the determination of antibiotic resistance has also been sought (Klein et al., 1998; Franz et al., 2001; Ben Omar et al., 2004) and is still being investigated (Delpech et al., 2012; Jahan et al., 2013; Camargo et al., 2014; Gousia et al., 2015; Ben Said et al., 2016). Many reviews of enterococci in foods and their antibiotic resistances have been conducted
Garg and Mittal; 1991; Giraffa et al., 1997; Franz et al., 1999, 2001, 2003, 2011; Robredo et al., 2000; Giraffa 2002, 2003; Foulquié Moreno, et al., 2006; Ogier and Serror, 2008). They are widely found in food, with meat and cheese being the main foods investigated. Antibiotic resistance is also widespread, with resistance to individual antibiotics occurring in varying proportions (Giraffa, 2002). Vancomycin-resistant enterococci are the main concern; however, other antibiotic resistances, such as erythromycin, tetracycline and chloramphenicol, have been flagged as being clinically important (Soares-Santos et al., 2015). Although enterococci have been used as indicators of faecal contamination, it is recognised that their presence in food may not be as a result of direct faecal contamination, as they are widespread in the environment (Foulquié Moreno et al., 2006).

Because of the risk of agricultural use of antibiotics favouring the emergence of antibiotic resistant enterococci (Bonten et al., 2001; Hammerum et al., 2010), the issue of the incidence of enterococci present in food and whether or not this poses a risk to human health has been raised in the international literature. Several studies have looked at the colonisation of the human gut after ingestion of antibiotic resistant enterococci as well as antibiotic resistance gene transfer within the human gut. This has been discussed in reviews on occasion (see Heuer et al., 2006; Hammerum et al., 2010; Hammerum, 2012). While the enterococci causing VRE outbreaks in hospitals are usually genetically different from those of animal, food or healthy human origin (Bonten et al., 2001; Heuer et al., 2006), similar Pulsed Field Gel Electrophoresis (PFGE) and Multi-Locus Sequence Typing (MLST) types of VRE have been found in human stools and animals (Hammerum, 2012), indicating that there is cross-over between animals and humans. It has been found that when humans have intentionally ingested monitored strains of enterococci, the enterococci have only transiently colonised the human gut when the participants were not receiving antibiotic treatment (Hammerum et al., 2010; Hammerum, 2012). A further study similarly found that probiotic strains of enterococci ingested in yoghurt only had transient colonisation of the gut (Pimentel et al., 2012). Nonetheless, it has been shown that the transfer of vancomycin resistance has occurred between E. faecium isolates within the human gut, although again the isolates were detected as having transient residence in healthy volunteers (Lester et al., 2006).
Although the residence of the enterococci in the gut was deemed to be transient (Lester et al., 2006), this could not be proven definitively. There is always the possibility that the VRE remain in the gut below the limits of detection, where they could re-emerge when conditions were favourable, such as in times of ill health or antibiotic treatment. Essentially, it is unknown absolutely what may happen to people if they ingest antibiotic resistant enterococci.

Enterococci have been investigated not only for their potential deleterious impacts on human health but also for the beneficial role that they may play in certain foods, particularly dairy products, as well as for other health benefits. They are found in a broad variety of European cheeses made from cow, goat, sheep and buffalo milk (Foulquié Moreno et al., 2006), and are included as starter cultures where they may be important for flavour development and ripening (Giraffa, 2002). Enterococci have also been registered as probiotic cultures, such as *E. faecium* SF68 and *E. faecalis* Symbioflor 1, for treatment of ailments such as diarrhoea, irritable bowel syndrome, high cholesterol and immune regulation in people (Franz et al., 2011). They are additionally used in animals to prevent and treat diarrhoea, exclude other gastrointestinal pathogens, improve growth performance and health in poultry, and growth performance and digestion in cattle (Franz et al., 2011). In addition, enterococci have potential uses in meat and cheese products due to the bacteriocins produced by some isolates which are active against *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus* and *Clostridium* (Hugas et al., 2003; Foulquié Moreno et al., 2006). It is this dual role as pathogen or beneficial microbe that causes debate (Franz et al., 2011). Furthermore, there can be uncertainty regarding the origin of enterococci in food, as pointed out in a study of farmhouse cheese where it was unknown how the milking equipment on the farm was initially contaminated by the enterococci and if the consuming the cheese was the source of the enterococci in the people (Gelsomino et al., 2002).
1.3 Traits of importance in occupying habitats

1.3.1 Antibiotic resistance
Although enterococci are described as having intrinsic resistance to a number of antibiotics, including penicillin, cephalosporins, and low levels of clindamycin and aminoglycosides, it is their acquired resistances that have brought further attention to this group of microorganisms (Murray, 1990a). Penicillin resistance, in particular, is 10-100 times greater in enterococci compared to most streptococci, with average Minimum Inhibitory Concentrations (MIC) between 2-8 µg/mL considered to be not clinically resistant, although higher level, acquired resistance can also develop (Murray, 1990a; NCCLS, 2003). Other acquired resistances include the antibiotics chloramphenicol, erythromycin, tetracycline, fluoroquinolones, high levels of aminoglycosides, lincosamides, linezolid, everninomicins and vancomycin (Murray, 1990a; Klare et al., 2003). From a medical treatment perspective, resistances to vancomycin and high-level resistances to aminoglycosides and penicillins are the most problematic, especially when these coincide in the same microorganism (Murray, 1997). Vancomycin is an antibiotic normally held in reserve to treat enterococci with resistance to ß-lactams and high level aminoglycosides (Bonten, 2001). It is this antibiotic that receives the most attention with regards to enterococci and the use of the term VRE is widespread.

Despite the growing number of *Enterococcus* species, enterococcal infections are predominantly caused by only two species, the original ones, *E. faecalis* and *E. faecium* (Rubinstein and Keynan, 2013). Occasionally other illnesses in humans have arisen due to *E. durans*, *E. gallinarum*, *E. casseliflavus* and *E. raffinosus* (Ogier and Serror, 2008). Originally, enterococcal infections were largely due to *E. faecalis* (Murray, 1997). With the rise of VRE infections, the proportions have been shifting (Arias and Murray, 2012). Prior to the early 1990’s, *E. faecalis* infections were due to the VanA genotype, with the VanB genotype being less common (Murray, 1997; Bonten et al., 2001). More recently, VRE infections in the US and Europe are still mostly due to enterococci with the VanA genotype (Rubinstein and Keynan, 2013; O’Driscoll and Crank, 2015); however, VRE
infections are now predominantly due to *E. faecium* (Arias and Murray, 2012; O’Driscoll and Crank, 2015).

Like many countries in the world that use antibiotics, VRE are present in Australian hospitals (Johnson et al., 2010; Coombs et al., 2014). A study in Melbourne investigated the presence of VRE in the stools of healthy individuals and found that only 0.2% of the stools contained VRE (Padiglione et al., 2000). Furthermore, the PFGE pattern of the two community VRE isolates differed to those that were more commonly found in Melbourne hospitals (Padiglione et al., 2000). This is consistent with other reports where the VRE found in hospital patients were genetically distinct from those found in the community and in animals (Bonten et al., 2001). Unlike VRE in other countries, though, the predominant VRE in Australia are caused by *vanB E. faecium*, not *vanA E. faecium* (Coombs et al., 2014), suggesting that Australia has a distinctive community of enterococci. The community of enterococci in Australian food has not been previously reported.

1.3.2 Gene transfer

In addition to the immediate treatment difficulties posed by antibiotic resistance in enterococci, a concern about antibiotic resistance in enterococci is their potential to transfer these resistances to other pathogenic bacteria. The transferability of antibiotic resistance amongst enterococci and from enterococci to other microorganisms has been examined in numerous studies. Tetracycline resistance has been transferred from *Streptococcus faecalis* (now *E. faecalis*) to *Staphylococcus aureus* either on a plasmid or transposon during *in vitro* experiments (Jones et al., 1987). In another *in vitro* experiment, multiple drug-resistant *E. faecalis* and *E. faecium* donors transferred gentamycin resistance to the plasmid and chromosome, respectively of *S. aureus* recipients (Noble et al., 1996). *S. aureus* has also been the recipient of vancomycin, erythromycin, streptomycin and chloramphenicol resistance from a multiply resistant *E. faecalis*, although tetracycline resistance failed to transfer in this experiment using a mouse skin model (Noble et al., 1992). In an experiment which utilised an enterococci isolate from food, an *E. faecalis* cheese isolate was used to transfer tetracycline resistance on a transposon to several bacteria, including *E. faecalis, Listeria innocua,*
*Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, *Leuconostoc mesenteroides* as well as *S. aureus* (Perreten et al., 1997). However, none of these experiments were conducted in a food model and the majority only looked at transfer from *E. faecalis*. The transfer of erythromycin resistance was achieved from *E. durans* isolated from fermented rice and lentils to *E. faecalis in vitro* (Thumu and Halami, 2014). In a reverse transfer experiment, a *L. lactis* dairy isolate was able to transfer tetracycline resistance to *E. faecalis in vitro* but not during yoghurt production (Toomey et al., 2009), indicating that the behaviour of bacteria in real food-based situations can be different to artificial situations in a laboratory. In an attempt to remedy the artificial situation *in vitro*, Cocconcelli et al. (2003) transferred both vancomycin and tetracycline resistance among enterococci during cheese and sausage manufacture. Both the donors and recipients in this experiment were *E. faecalis*. As this species has an efficient system for exchanging genetic material (Clewell et al., 2002), this result is not unexpected. However, the majority of *E. faecalis* isolates from dairy and raw meat were unsuccessful in the transfer of antibiotic resistance to *E. faecalis* using other in vitro mating experiments (Wilcks et al., 2005; Hummel et al., 2007), showing that the ability to transfer antibiotic resistance genes is not universally possible and is dependent on the genetic mechanisms within individual enterococci.

### 1.3.3 Virulence

In addition to antibiotic resistance, enterococci possess a number of virulence factors that that contribute to their ability to cause disease. These have been discussed at length in many reviews including the following: Giraffa et al. (1997), Franz et al. (2003), Kayser (2003), Foulquié Moreno et al. (2006), Sava et al. (2010), Franz et al. (2011), Arias and Murray (2012). Virulence factors encompassing sex pheromones, adhesion factors and secreted virulence factors in enterococci were considered as possible traits to investigate in this study and are discussed here.

#### 1.3.3.1 Sex pheromones

*E. faecalis* has an efficient way in which to exchange genetic information via conjugative plasmids. A plasmid-free isolate secretes a multiple peptide sex pheromone that stimulates a plasmid-containing isolate to secrete Aggregation Substance (AS). The
aggregation substance binds to the “enterococcal binding substance” on the recipient, causing clumping and facilitates transfer of a copy of the plasmid to the recipient (Clewell et al., 2002). This method of transfer usually solely occurs between *E. faecalis* isolates, and occurs only seldomly in *E. faecium* (Wirth, 2000).

### 1.3.3.2 Adhesion factors

Aggregation Substance, which is encoded on pheromone-responsive plasmids, is an adhesin that promotes clumping of enterococci cells, and facilitates adhesion and invasion of host cells (Franz et al., 2011; Arias and Murray, 2012). Enterococcal Surface Protein (Esp) is a cell-wall associated protein that has structural similarities with the *S. aureus* biofilm-associated protein BAP and can act as a colonisation factor in urinary tract infections (Archimbaud et al., 2002), and affects the pathogenesis of endocarditis in experimental models (Arias and Murray, 2012). It has also been shown to promote biofilm formation of *E. faecalis* on abiotic surfaces (Toledo-Arana et al., 2001). Adhesin of collagen (Ace) is an enterococcal MSCRAMM (microbial surface components recognising adhesive matrix molecules) important in early stages of infection (Arias and Murray, 2012). The Ace binds to collagen and laminin, is similar to the collagen-binding protein Cna of *S. aureus* (Franz et al., 2003) and may contribute to the pathogenesis in endocarditis (Koch et al., 2004). Named for *E. faecalis* antigen A (Singh et al., 1998), *E. faecalis* and *E. faecium* each have individual adhesin genes, *EfaA*<sub>fs</sub> and *EfaA*<sub>fm</sub>, respectively, which act as endocarditis antigens (Franz et al., 2003; Franz et al., 2011). Using animal models, *EfaA*<sub>fs</sub> has been shown to influence pathogenicity (Singh et al., 1998).

### 1.3.3.3 Secreted virulence factors

The β-haemolysin cellular toxin is a confirmed virulence factor (Franz et al., 2003). It enhances virulence in animal models (Franz et al. 2011) and can lyse human, horse and rabbit blood cells (Arias and Murray, 2012). It has been shown to increase the risk of death due to bacteraemia in humans, although it is not a prerequisite for virulence (Franz et al., 2003). Haemolysin is encoded by the gene *cyl* (Arias and Murray, 2012). Gelatinase is a metalloendopeptidase which can hydrolyse gelatin, collagen and haemoglobin, and activates autolysin, leading to biofilm formation (Foulquié Moreno et
al., 2006; Arias and Murray, 2012). It can cleave fibrin, damaging host tissue and allowing the bacteria to spread (Franz et al., 2011).

1.3.3.4 Final selection of virulence factors

In a study reported by Eaton and Gasson (2001), which investigated the presence of four sex pheromone-encoding genes, each of the 20 widely-sourced *E. faecalis* and none of the 26 *E. faecium* isolates tested possessed the pheromone. As these results were so conclusive, searching for the presence of such genes was considered not to be a priority in the current study. Of the above mentioned adhesion factors, AS and Esp have been studied more and offer a better point of reference than Ace and EfaA. Additionally, EfaA was present in 94 % of widely sourced enterococci (Eaton and Gasson, 2001), making the search for this adhesin redundant; therefore, AS and Esp were the adhesins selected for investigation. The β-haemolysin and gelatinase virulence factors both act on host tissue (Koch et al., 2004) and are simple to detect phenotypically, so were both selected for testing in the current study. Furthermore, it has been suggested that major enterococcal virulence factors are β-haemolysin, gelatinase and AS (Franz et al., 2011), adding support to limiting the investigation of these in the current study.

1.3.4 Heat resistance

Enterococci have been isolated from food after heat treatment (Batish and Ranganathan, 1984a; Magnus et al., 1986). Although this could be due to post processing contamination, it is also potentially due to the presence of the enterococci in the raw product and survival through the cooking process. Enterococci have been described as being able to survive pasteurisation (Giraffa et al., 1997), however this has not been quantified. Batish et al. (1985) looked at the ability of enterococci to survive batch pasteurisation (63 °C/30 min) but not High Temperature/Short Time (HTST) pasteurisation (72 °C/15 s), which is the normal minimum pasteurisation procedure in Australian dairy factories (FSANZ, 2004). Several studies have measured the heat resistance of enterococci (Magnus et al., 1986; Kearns et al., 1995; Renner and Peters, 1999; Spinks et al., 2006; Aguirre et al., 2009) but have not investigated HTST conditions. Investigations to quantify enterococci survival at milk HTST pasteurisation conditions
would be of use to the dairy industry to inform processors of potential contamination risks following pasteurisation.

1.4 Aims of this thesis

The climate and agricultural practices specific to south-eastern Australia may have an influence on the enterococci that are in this environment. This in turn will influence the enterococci that eventuate in the food supply, which has not previously been assessed in Australia. Research described in this thesis focused on the dairy industry in SE Australia, investigating the prevalence and species of enterococci in raw milk and dairy products in relation to the seasons over a calendar year. The survey was conducted in 2002-2003, in part-time studies. This type of work had not been done in Australia prior to this time and up to the present time, so the data can be utilised as a baseline to temporal studies.

The heat resistance of the predominant species obtained in the survey was established, as this can impact what enterococci are able or likely to be found in products following HTST pasteurisation. Following on from this, the ability of thermoduric enterococci to survive and grow in dairy product environments was investigated. As the presence of antibiotic resistance in food enterococci is of prevailing concern, the phenotypic resistance to medical and veterinary antibiotics was investigated along with strategic virulence factors, and the genetic basis and antibiotic resistance transfer of medically significant resistances examined. The persistence of enterococci in raw milk silos was investigated, since a persistent population could result in recurrent contamination. Relationships between antibiotic resistance and the ability to form biofilms were investigated, as biofilms may enable the persistence of bacteria within food processing environments. Finally, how the prevalence, heat resistance, persistence, antibiotic resistance and gene transfer capability of dairy enterococci interact was evaluated in relation to assess the risk of enterococci in the dairy food chain and hence the safety of Australian dairy products.
CHAPTER 2 - SURVEY OF THE LEVEL, SPECIES DISTRIBUTION AND SEASONAL OCCURRENCE OF ENTEROCOCCI IN RAW MILK AND DAIRY PRODUCTS
2.1 Introduction

Enterococci are widespread throughout the environment (Giraffa, 2002), are inhabitants of mammalian intestines (Stiles, 1989) and have been isolated from plants and soil (Franz et al., 1999). Enterococci have been isolated from dairy cow feces (Batish and Ranganathan, 1984b; Gelsomino et al., 2001) but not always from every cow tested (Kagkli et al., 2007). The survival of enterococci in feces has been shown to be longer than other fecal indicators (Sinton et al., 2007). Enterococci, along with *E. coli*, are used as indicators of fecal contamination in watercourses (Hampson et al., 2010) as well as in food (Stiles, 1989) and can serve as indicators of process hygiene and food and drinking water quality (Halkman and Halkman, 2014). While recognized as conferring beneficial properties to fermented milk products, if enterococci are present in dairy foods post-manufacture they may pose food safety risks as potential pathogens and reservoirs of antibiotic resistance, and contribute to food spoilage (Giraffa et al., 1997; Ogier and Serror, 2008; Franz et al., 2011). Consequently, identifying sources of enterococcal contamination in milk supplies, and assessing risk factors of and persistence in manufactured products have received increasing attention in the literature (for example, see Buhnik-Rosenblau et al., 2013; Hammad et al., 2015).

Enterococci can enter milk from human or animal feces, water sources, the farm environment, or from milking equipment, bulk storage tanks and equipment used during milk harvesting or local processing (Gelsomino et al., 2001, 2002). The occurrence of enterococci in raw bovine milk has been explored in several countries, where *E. faecalis* was the predominant species found in studies from India, the Czech Republic and Turkey (Batish and Ranganathan, 1984b; Schlegelova et al., 2002; Citak et al., 2005) but *E. faecium* was the main raw milk species in a South African study (Wessels et al., 1988). In contrast, an Irish farmhouse study found that *E. casseliflavus* and *E. faecalis* were most frequently isolated in raw milk from a small herd of 27 cows (Gelsomino et al., 2001). These species were also found in human feces, cheese made from the raw milk, tap water and milking equipment, whereas *E. faecium* dominated cow feces when isolated at all. It was concluded (Gelsomino et al., 2002) that milking equipment was the
probable source of milk contamination, where *E. casseliflavus* persisted in the machine and bulk tank on the one farm examined. A study has looked at the seasonality of bovine fecal indicator organisms, including enterococci, on New Zealand farms, over one year (Sinton et al., 2007). The study found that although presumptive enterococci counts in feces were variable, the highest counts occurred in spring (Sinton et al., 2007), the peak milk harvesting season.

Although numerous studies have identified the presence of enterococci in raw and pasteurised bovine milk, there have not been longitudinal studies on the ecology of enterococci in bovine milk which have examined the seasonality of occurrence and changes of species over time. Seasonality is of particular interest to the dairy industry in Australia, as dairy production occurs in regions that vary significantly in maximum daily temperatures, climatic conditions (Australian Government, 2015) and nature of water supply. Victorian production of cow’s milk is 66 % of national production (9.5 million tonnes), representing ~1.5 % of world production (AHDB, 2015), and occurs in three major dairying regions to the north, east and west of the capital city, Melbourne (Victorian Government, 2014). The northern and southern regions experience significant differences in monthly maximum and minimum temperatures (Supplements S1 and S2 in Appendix E) and water supply varies from rain-fed only, irrigation and ground-water supplies (Victorian Government, 2014). Given that several factors may influence the nature of the microbiota in raw milk (region, climate, water supply, transport distance to manufacturing plants and temperature of transport), a survey was undertaken to determine whether regionality, season of milk harvest (sampling over one year) and location of manufacturing plants influenced the species type and prevalence of enterococci in raw milk as delivered to bulk holding facilities in manufacturing plants and dairy products obtained from the manufacturers: to our knowledge, this is the first survey of this type in Australia.

Prior to the commencement of the survey, media were evaluated for their suitability in enumerating all of the enterococci species likely to be present in milk samples. Detection of enterococci in food samples using cultural techniques can be problematic given that some microbiological media permit the growth of related groups of bacteria
that interfere with results and that the more selective media can inhibit the growth of some of the newly described enterococci species (Devriese and Pot, 1995). Furthermore, the confirmation of presumptive enterococci from selective isolation agar is difficult, as there are no phenotypic or physiological characteristics that separate all enterococci from other Gram-positive, catalase negative cocci. This is a result of the identification of new enterococci species that do not exhibit the classical phenotypical Enterococcus traits, including the presence of the Lancefield group D antigen and the ability to grow at 10 and 45 °C, in the presence of 6.5 % NaCl and at pH 9.6 (Giraffa, 2000; Giraffa, 2014).

The current Australian and International Standards for the microbiological examination of food do not include methods for the detection of enterococci. Standard methods exist for the isolation of enterococci from water (Standards Australia, 1995a, AS 4276.8; 1995b, AS 4276.9) but raw milk and dairy products contain different microbiota than water. Therefore it cannot be assumed that the media used for water is appropriate for dairy foods. A method for the detection of enterococci is described in the Compendium for the Microbiological Examination of Foods (Hartman et al., 1992). This utilizes KF Streptococcus agar, however it recommends that a more selective agar may be required for the testing of dairy products. An earlier review found that more than 12 different media have been used for the selective isolation of enterococci in dairy foods and that greater selectivity negatively impacted recovery rates (Domig et al., 2003). Current studies have used a variety media, including Kanamycin Aesculin Azide Agar, Bile Esulin Azide Agar, Fluorescent Gentamicin Thallous Carbonate Agar, Slanetz and Bartley Agar and Enterococcosel Agar (Belicová et al., 2007; Fracalanzza et al., 2007; Delpech et al., 2012; Hammad et al., 2015) and comparative data for the performance of enterococci media are still lacking for dairy foods. Consequently, a preliminary study was undertaken to compared the ability of commercially available enterococci media to recover and differentiate enterococci, using American Type Culture Collection (ATCC) reference and wild-type isolates.
2.2 Materials and Methods

2.2.1 Media assessment strains

A selection of wild-type enterococci and related species, characterised in previous studies at Food Science Australia and described in the literature to give false positive results on enterococci selective media (Manafi, 2000), were used for the assessment of media. The enterococci were isolated previously from raw milk obtained from farm pick-up tankers in the eastern, northern and western regions of Victoria in 2001, Factories Q, R and S, respectively. The milk samples had been obtained in the first week of January, in mid-summer. The strains had been isolated from Enterococcosel agar (BBL, Becton Dickinson, Sparks, Maryland, USA) incubated at 35 °C for 48 h. Presumptive isolates were previously confirmed as enterococci if they were catalase negative, Gram-positive and positive for pyrrolidonyl aminopeptidase (PYR) (Chapter 2.2.6.5), leucine aminopeptidase (LAP) and arginine dehydrolase (ADH), and speciated (Chapter 2.2.6.6). The LAP and ADH enzymes were assessed using Rosco Diatabs® (Dutec Diagnostics, Croydon, NSW, Australia) following the manufacturer’s instructions. Reference strains of Enterococcus spp. (ATCC) were obtained from bioMérieux (Microbiologics Inc., St. Cloud, Minnesota, USA) (Table 2.1).

2.2.2 Maintenance and storage of cultures

Cultures were stored long term on cryobeads (Microbank™, Pro-Lab Diagnostics, Austin, Texas, USA) and in Nutrient Broth (NB; Oxoid, Basingstoke, Hampshire, England) with 20 % glycerol (BDH, Kilsyth, Victoria, Australia) put into cryotubes (Greiner bio-one, Frickenhausen, Germany) and stored at -70 °C.
Table 2.1 Strains used to evaluate enterococci media

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Reference or lab number</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. avium</em></td>
<td>ATCC 14025</td>
<td>bioMérieux</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>ATCC 19433</td>
<td>bioMérieux</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>ATCC 29212</td>
<td>bioMérieux</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>ATCC 51299</td>
<td>bioMérieux</td>
</tr>
<tr>
<td><em>E. gallinarum</em></td>
<td>ATCC 35667</td>
<td>bioMérieux</td>
</tr>
<tr>
<td><em>E. hirae</em></td>
<td>ATCC 8043</td>
<td>bioMérieux</td>
</tr>
<tr>
<td><em>Aerococcus viridans</em></td>
<td>ATCC 700406</td>
<td>bioMérieux</td>
</tr>
<tr>
<td><em>E. casseliflavus</em></td>
<td>1987r</td>
<td>raw milk, FSA</td>
</tr>
<tr>
<td><em>E. casseliflavus</em></td>
<td>2003V1</td>
<td>raw milk, FSA</td>
</tr>
<tr>
<td><em>E. durans</em></td>
<td>1998g</td>
<td>raw milk, FSA</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>1967g</td>
<td>raw milk, FSA</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>1973r</td>
<td>raw milk, FSA</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>2000b</td>
<td>raw milk, FSA</td>
</tr>
<tr>
<td><em>E. gallinarum</em></td>
<td>1975V1</td>
<td>raw milk, FSA</td>
</tr>
<tr>
<td><em>E. hirae</em></td>
<td>1981g</td>
<td>raw milk, FSA</td>
</tr>
<tr>
<td><em>E. malodoratus</em></td>
<td>1978r</td>
<td>raw milk, FSA</td>
</tr>
<tr>
<td>Enterococcus sp. unknown</td>
<td>1970g</td>
<td>raw milk, FSA</td>
</tr>
<tr>
<td><em>Pediococcus</em>&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1965V1</td>
<td>raw milk, FSA</td>
</tr>
<tr>
<td>Gram-positive short rods</td>
<td>1977 EV1</td>
<td>raw milk, FSA</td>
</tr>
<tr>
<td>Gram-positive large cocci</td>
<td>1990 EV1</td>
<td>raw milk, FSA</td>
</tr>
<tr>
<td>False positive non-enterococci</td>
<td>1990b</td>
<td>raw milk, FSA</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> sp lactis</td>
<td>FSA 2042</td>
<td>FSA culture collection</td>
</tr>
</tbody>
</table>

<sup>1</sup> FSA = Food Science Australia  
<sup>2</sup> presumptive identification
Isolates were resuscitated from cryogenic beads by inoculating into Brain Heart Infusion Broth (BHIB; Oxoid) and incubating at 37 °C for 18-24 h. Turbid cultures were then streaked onto Brain Heart Infusion Agar (BHIA) slopes (Oxoid) and again incubated at 37 °C for 18-24 h. The slopes were used as working cultures and were stored at 4 °C for up to 3 months. Cultures were also streaked from BHIB onto BHIA plates to check purity and viability.

Reference strains used to assess media were inoculated straight onto Nutrient Agar (NA; Oxoid) slopes from the commercial swab applicators. The slopes were stored at 4 °C and used as working cultures for two months. Cultures were also streaked from BHIB onto BHIA plates to check purity and viability.

2.2.3 Assessment of commercial media

Two main groups of media were identified for the selective detection of enterococci: those containing bile plus esculin and those containing sodium azide plus triphenyl tetrazolium chloride (TTC). Two formulations were evaluated from each group. In the bile/esculin group, Enterococcus agar containing 1 % bile and 0.1 % esculin (ECA), (BBL) and Bile Esclin agar containing 2 % bile and 0.1 % esculin (BEA) (Oxoid) were selected. From the sodium azide/ TTC group, KF Streptococcus agar (KF-Strep), (Amyl Media, Dandenong, Victoria, Australia) and m Enterococcus agar (MEA) (BBL), both containing 0.04 % sodium azide and 0.01 % TTC, were selected. The KF-Strep also contained 0.0015 % of the selective indicator bromocresol purple. Two other media were assessed, including a chromogenic medium: Chromocult Enterococci broth (CEB), (Merck, Darmstadt, Germany) and MacConkey agar No. 2 (Mac2), (Oxoid). The CEB contained 0.06 % sodium azide and 0.004 % X-GLU (5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside), which is a chromogenic indicator for growth of enterococci. Since CEB was only available in broth format in Australia at the time of the commencement of work (2002), Bacteriological Agar No. 1 (1.5 %, Oxoid) was added to the broth to make agar plates (CEBA). The Mac2 contained 0.15 % bile salts, 0.005 % neutral red and 0.0001 % crystal violet. Media were prepared according to manufacturer’s instructions. Autoclaving was conducted for a time commensurate with the volume of media...
prepared as previously evaluated for the autoclaves at Food Science Australia, Werribee to achieve a core temperature of 121 °C for 15 min (Appendix F).

Commercial media were assessed to determine their ability to differentiate and recover enterococci. Isolates were streaked onto the enterococci media for the assessment of colony morphology (Chapter 2.2.3.2) and diluted cultures were spread onto media (Chapter 2.2.3.3) to compare recovery with growth on BHIA, a non-selective, nutritious growth medium.

2.2.3.1 Growth temperature
Colony morphology studies were conducted at both 35 and 37 °C. Media manufacturers for ECA, KF-Strep, MEA and CEBA recommended growth at 35 °C. The recommended growth temperature for BEA was 37 °C (Oxoid) or 35 °C (BBL), while a temperature was not indicated for Mac2. However, the Australian Standards (AS1766) for testing mesophilic microorganisms in foods is generally either 30 or 37 °C, with potential human pathogens and enteric indicator organisms tested at 37 °C. In addition, the Australian Standards for water testing indicate that enterococci can be evaluated on BEA and MEA at either 35 or 37 °C (Standards Australia, 1995a, AS 4276.8; 1995b, AS 4276.9). As colony morphology at 37 °C did not differ to the morphology at 35 °C, it was decided that 37 °C would be a more appropriate and convenient incubation temperature. Therefore experiments to recover enterococci were routinely conducted at 37 °C.

2.2.3.2 Colony morphology
The strains were inoculated from NA slopes into BHIB and incubated normally for 16 h or for longer if sufficient turbidity was not reached after 16 h. Cultures were streaked onto BHIA, ECA, BEA, KF-Strep, MEA, CEBA and Mac 2 and incubated for 48 h. Media reactions were recorded, noting colony size and colour.

2.2.3.3 Recovery of enterococci
Commercial enterococci media were assessed for the ability to recover known concentrations of microorganisms compared with the non-selective control medium, BHIA. Strains were inoculated from BHIA slopes into BHIB and incubated at 37 °C for
16 h. Direct Microscopic Counts on the diluted \(10^{-2}\) 16 h broths (Standards Australia, 1994, AS1766.3.10) were determined and the cultures were diluted to \(10^2\) cfu/mL. Diluted cultures were spread plated (0.1 mL) onto the media (Standards Australia, 1991, AS1766.1.4), in triplicate, and incubated at 37 °C for 48 h then the number of colonies on plates was recorded.

### 2.2.4 Modification of commercial media

#### 2.2.4.1 Modified formulations and colony morphology

The CEBA medium was selected for modification potentially to improve selectivity for enterococci. The TTC was added to the CEBA at two levels, 0.01 % and 0.005 %. Both the TTC levels were tested with and without 1.0 % sodium glycerophosphate (SGPP) (Amyl Media) (Table 2.2). Colony morphology was recorded for all four of the modified formulations (CEBA 1, 2, 3 and 4). Then CEBA 2 was selected for further evaluation and was compared with BHIA, MEA and CEBA for differentiation and recovery of enterococci, employing the method described previously (Chapter 2.2.3.2 and Chapter 2.2.3.3).

#### 2.2.4.2 Differentiation of species

Mixtures of \(E.\ faecalis, E.\ faecium, E.\ durans, E.\ casseliflavus, E.\ gallinarum, E.\ hirae, E.\ malodoratus, A.\ viridans\) and the wild-type Gram-positive rod 1977 EV1, diluted to \(10^2\) cfu/mL, were spread plated (0.1 mL each) in selected pairs onto BHIA, MEA, CEBA and CEBA 2 and incubated at 37 °C for 48 h. Pairs were selected to obtain a range of colony morphologies on the same media. Media were assessed for their ability to differentiate enterococci species and to discriminate between enterococci and non-enterococci in mixed culture.

#### 2.2.4.3 Recovery on modified media

The CEBA 2 medium was selected for further evaluation and was compared with BHIA, MEA and CEBA for differentiation and recovery of enterococci and non-enterococci, employing the methods described previously (Chapter 2.2.3.2 and Chapter 2.2.3.3).
2.2.5 Dairy sample acquisition

2.2.5.1 Milk

Raw bovine milk was sampled 12 times at approximately monthly intervals, from April 2002 until February 2003, from three different bulk raw milk silos at each of six dairy factories in Victoria, Australia (n = 211) (Table 2.3). Dairy factories were located in Victoria’s three dairying regions, east (Factories A and B), north (Factories C and D) and west (Factories E and F) of Melbourne, the capital of Victoria. All of the factories were within a 200 km radius of Melbourne and the distance between the centers of the regions was approximately 250 km. Samples were assigned to a season based on the month they were collected (Summer: December, January, February; Autumn: March, April, May; Winter: June, July, August; Spring: September, October, November). Milk was not collected from Factory C in July (winter) and only two milk samples were collected from Factory A in December (summer) and from Factory C in February (summer). Factory pasteurised milk (72 °C, 15 s) from two processing lines at Factory B was sampled approximately at monthly intervals over a year (n = 24). Milk samples were placed into insulated containers with either ice packs or crushed ice, transported to the CSIRO Melbourne laboratory by overnight courier and stored at < 4 °C. All testing was performed on the day of sample arrival.

2.2.5.2 Butter

Two 500 g packaged butter samples were obtained quarterly from Factory G in western Victoria, and from Factory H in eastern Victoria, for a total of four samples per quarter (Table 2.4). The butter was transported overnight in insulated containers either with freeze bricks or ice and stored in the laboratory at < 4 °C prior to testing on the day of sample arrival. Butter samples in spring and samples from the west in summer were unsalted, while the remainder were salted.

2.2.5.3 Cheddar cheese

Cheddar cheese, made with mesophilic starter cultures in pasteurised milk, and supplied in approximately 5 kg blocks, was obtained from Factory M in a state other than Victoria (Table 2.4). Australian Cheddar cheese typically has 35-36.5 % moisture and a pH 5.2-5.3
Table 2.2 Modified media formulations

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
<th>Addition to Chromocult Enterococci agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromocult 1</td>
<td>CEBA 1</td>
<td>0.01 % TTC</td>
</tr>
<tr>
<td>Chromocult 2</td>
<td>CEBA 2</td>
<td>0.005 % TTC</td>
</tr>
<tr>
<td>Chromocult 3</td>
<td>CEBA 3</td>
<td>0.01 % TTC + 1.0 % SGPP</td>
</tr>
<tr>
<td>Chromocult 4</td>
<td>CEBA 4</td>
<td>0.005 % TTC + 1.0 % SGPP</td>
</tr>
</tbody>
</table>

Table 2.3 Raw and pasteurised milk sample receival schedule

<table>
<thead>
<tr>
<th>Season</th>
<th>Sampling Date</th>
<th>East¹</th>
<th>North¹</th>
<th>West¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autumn</td>
<td>16 April</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>7 May</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>28 May</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Winter</td>
<td>18 June</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>9 July</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>14 August</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Spring</td>
<td>11 September</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>17 October</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>14 November</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Summer</td>
<td>3 December</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>14 January</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>11 February</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

¹Region
²Factory

Table 2.4 Dairy product sample receival at each factory

<table>
<thead>
<tr>
<th>Sampling season¹</th>
<th>Butter G²</th>
<th>Milk powder H²</th>
<th>Cheese J²</th>
<th>K²</th>
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<td>Summer</td>
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¹Samples were received at the end of each season
²Factory
(Victorian College of Agriculture and Horticulture, 1993). Two blocks of cheese were obtained quarterly and transported overnight in insulated containers either with freeze bricks or ice. The cheese was stored at 4 °C prior to testing on the day of arrival.

2.2.5.4 Milk powder

Fourteen bulk milk powders (500 g) were obtained from Factory J in western Victoria and Factory K in eastern Victoria in autumn, and from Factory J and Factory L for winter, spring and summer (Table 2.4). Factory L was located in a state other than Victoria. The aim was to test four milk powder samples each quarter, with two each from two factories. However, only one powder sample was received in winter and spring from Factory L. For every quarter, Factory J supplied two samples of low heat skim milk powder. In autumn, Factory K supplied one low-heat skim milk powder sample and one full cream milk powder sample. Factory L supplied one low heat skim milk powder sample in winter and spring and two low heat skim milk powder samples in summer. Milk powder was stored at room temperature prior to testing on the day of arrival.

2.2.6 Analysis of dairy samples

CEB or double-strength CEB (2XCEB) and CEBA were used in the detection of enterococci in the milk and dairy products. When necessary, serial dilutions of the milk (1 in 9 mL) were made in 0.1 % Bacteriological Peptone (Oxoid) (Standards Australia, 1991, AS1766.1.2).

2.2.6.1 Enumeration of enterococci in raw milk

On arrival, raw milk samples were serially diluted in 0.1 % Bacteriological Peptone (Standards Australia, 1991, AS1766.1.2) and 0.1 mL (10⁰ to 10⁻² dilutions) was spread onto CEBA, in duplicate (Standards Australia, 1991, AS1766.1.4). All broths and plates were incubated at 37 °C for 48 h. Presumptive enterococci colonies on the spread plates were enumerated (Standards Australia, 1991, AS1766.1.4), counting all blue colonies with morphology typical of enterococci as compared to the *E. faecalis* reference strain ATCC 29212. The limit of detection of enterococci by plate counts was 10 cfu/mL.
2.2.6.2 Detection of enterococci in pasteurised milk

Pasteurised milk (0.1 mL) was spread in duplicate onto CEBA without dilution, then incubated and enumerated as above. Because numbers surviving factory pasteurisation (72 °C, 15 s) were expected to be low and potentially undetectable on plate counts, enrichment in CEB was undertaken using three inoculation strategies for all pasteurised milk samples, with the following ratios of milk to CEB: 1 mL into 10 mL CEB; 10 mL into 10 mL of 2XCEB; and 100 mL into 100 mL 2XCEB. These enrichments had minimum detection levels of 1, 0.1 and 0.01 cfu/mL, respectively. All enrichment broths were streaked onto CEBA plates to detect presumptive enterococci. The broths and plates were incubated at 37 °C for 48 h.

2.2.6.3 Detection of enterococci in butter, Cheddar cheese and milk powder

For butter, Cheddar cheese and milk powder, 10 and 100 g of product were added to 90 and 900 mL of single strength CEB respectively, warmed to 45 °C and held at 45 °C for 15 min after homogenisation, prior to incubation. Butter was melted at 45 °C prior to dispensing into 10 and 100 g samples, which were homogenised by stomaching (Lab Blender 400, Seward, London, UK) for one and three minutes, respectively. All Cheddar cheese samples were homogenised by stomaching for three minutes. Milk powder samples were homogenised by stomaching for one minute. Following incubation, the enrichment broths were streaked onto CEBA as well as CEBA containing 6 µg/mL vancomycin (Sigma, St. Louis, Missouri, USA) (VCEBA). Butter, Cheddar cheese and milk powder enrichment broths were incubated at 37 °C for 72 h. Plates were incubated at 37 °C for 48 h.

2.2.6.4 Selection of isolates for confirmation as enterococci

Representative isolates were obtained using a Harrison's disc to randomly select colonies on spread plates (Harrigan and McCance, 1976), selecting the square root of the count on the plate. If the spread plates for pasteurised milk samples contained less than 20 enterococci colonies, two typical isolates were selected from the streak plates of the enrichment broths. Two typical isolates were selected from the streak plates of the butter, Cheddar cheese and milk powder enrichment broths. All selected isolates were sub-cultured into 5 mL of CEB and incubated at 37 °C until turbid and the colour of the
broth had changed from clear yellowish to blue. CEB cultures were then streaked onto BHIA plates and slopes, and incubated at 37 °C for 18 to 24 h. Plates were checked for purity and colonies re-streaked up to two times, if necessary, to obtain pure cultures.

### 2.2.6.5 Confirmation of isolates as enterococci

All selected isolates from both the raw and pasteurised milk were confirmed as enterococci using tests for catalase reaction, pyrrolidonyl aminopeptidase (PYR) reaction and growth at 45 °C and in 6.5 % NaCl. Enterococci had a negative catalase reaction, determined by the lack of bubble formation when colonies were emulsified in 30 % hydrogen peroxide (BDH, Kilsyth, Victoria, Australia). The PYR reaction was determined using Rosco Diatabs® (Dutec Diagnostics, Croydon, NSW, Australia) added to a McFarland Standard 4 culture suspension in 0.85 % saline, incubated at 37 °C for 18 to 24 h. Enterococci were PYR positive, showing a red reaction. A McFarland Standard 4 culture suspension was spotted onto a BHIA agar plate and incubated at 45 °C for 24 to 48 h. A 10 µL loop of the McFarland Standard 4 culture suspension was added to BHIB adjusted to 6.5 % NaCl and incubated at 37 °C for 24 to 48 h. Visible growth on the plate at 45 °C and in 6.5 % NaCl was characteristic of enterococci.

### 2.2.6.6 Speciation of isolates as enterococci

Identification of enterococci species was performed using the biochemical key developed by Manero and Blanch (1999), testing for carbohydrate utilisation in 2 mL of Phenol Red broth (Amyl Media) containing 5 % carbohydrate. Inoculated carbohydrate broths were incubated at 37 °C for 24 h, following the method of MacFaddin (1980). All seven carbohydrates of the biochemical key were included for the first three milk sampling periods. These were L-arabinose, α-methyl-D-glucopyranoside, mannitol, D-raffinose, ribose, sucrose (Sigma), and sorbose (Calbiochem, La Jolla, California, USA). After the third period, the utilisation of raffinose and sucrose was tested only when specifically required, as these carbohydrates were usually not necessary for the speciation of the isolates obtained. The majority of isolates required testing for arginine dehydrolase (ADH). This was performed following the method of MacFaddin (1980), using L-arginine (Sigma) and Decarboxylase Base Moeller (Difco, Sparks, Maryland, USA). The ADH tests were overlayed with paraffin oil (Sigma Pharmaceuticals, Clayton,
Victoria, Australia). *E. faecalis* ATCC 29212 was used as a positive control culture to verify the performance of all batches of media. It was also used as an example of *Enterococcus* in the confirmation tests.

### 2.2.7 Data analyses

Raw milk counts were transformed to $\log_{10}$ and analysed using Analysis of Variance to determine significant differences ($p < 0.05$) between the number of enterococci in samples from the factories, regions and seasons (least significant differences, LSD) with Genstat Release 6.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, Hertfordshire, UK). The average number of enterococci in the raw milk was determined by calculating the geometric mean using a $\log_{10}$ transformation. Counts that were 0 on the plate (< 10 cfu/mL) were analysed as 1 cfu/mL. The prevalence of each species in the raw milk was determined by calculating the percentage of each species out of the total number of enterococci isolated. The ANOVA tables are located in Appendix G, as supplements S3 and S4.

For the pasteurised milk, butter, Cheddar cheese and milk powder enriched samples, results were recorded for the smallest sample size in which enterococci were detected. This was presence in 1, 10 or 100 mL, or not detected in 100 mL enrichments. In the butter, Cheddar cheese and milk powder, it was presence in 10 or 100 g, or not detected in 100 g. The prevalence of each species of enterococci in each sample type was determined by calculating the percentage of samples containing each species.
2.3 Results

2.3.1 Assessment of commercial media

2.3.1.1 Colony morphology
2.3.1.1.1 Bile/Esculin group

The ECA and BEA produced results (grey colonies with black halos due to esculin hydrolysis) for most of the enterococci species. Most colonies of enterococci on ECA and BEA were 1-3 and 1-2 mm in diameter respectively. However, the *E. gallinarum* ATCC 49573 reference strain had decreased blackening on the agar and colonies less than 1 mm in diameter on both of these media, with atypically transparent colonies on ECA. The *E. avium* ATCC 14025 reference strain and the *E. hirae* 1981g, *E. casseliflavus* 1987r and *E. casseliflavus* 2003V1 wild-type isolates produced colonies 1 mm or less in diameter, mostly with negligible esculin reactions on ECA while the *E. hirae* 1981g wild-type isolate also had this morphology on BEA. Strains that produced typical esculin reactions caused blackening of the entire plates when numerous colonies were on the plates, obscuring reactions for individual colonies. The non-enterococci strains had mixed reactions on the media containing bile and esculin. While the *Pediococcus* isolate and the *A. viridans* reference strain had pale grey colonies, less than 1 mm in diameter with decreased black halos on BEA, they did not grow on ECA. On both media, the wild type isolate Gram-positive rod 1977 EV1 produced colonies similar to the wild-type enterococci mentioned above. The Gram-positive coccus 1990 EV1 wild-type isolate had atypical colonies, which were less than 0.5 mm on ECA, but it was barely visible on BEA. False positive 1990b had colonies less than 1 mm. In contrast, the *L. lactis* isolate did not grow on ECA and had white, punctiform colonies on BEA.
2.3.1.1.2 Sodium azide/TTC group

Reference strains and wild-type isolates of *E. faecalis* and *E. faecium* grew to 1.25 to 2 mm on KF-Strep, whereas the potential false positive, non-enterococci isolates were less than 1 mm in diameter. However, the *E. gallinarum* ATCC 49573 and *E. avium* ATCC 14025 reference strains and the *E. faecalis* 1967g, *Enterococcus sp.* 1970g, *E. hirae* 1981g, *E. casseliflavus* 1987r, *E. durans* 1998g and *E. casseliflavus* 2003V1 wild-type isolates were also 1 mm or less in diameter. Typical colonies on this media are pink to red and 0.5 to 2 mm in diameter. One of the other problems with the media was that, having followed the manufacturer’s preparation instructions, the media did not produce the typical red enterococci colonies required of the media. Instead, the agar surrounding enterococci colonies turned yellow, which then dissipated after overnight storage of the plates at 4 °C. Typical colonies on MEA were also expected to be pink to red, due to the incorporation of TTC in the media. While the *E. faecalis* and *E. faecium* isolates generally had pink to red colonies, 1-1.5 mm in diameter, the enterococci isolates that were smaller on KF-Strep were also smaller on MEA, as were *E. faecalis* 1967g, *E. casseliflavus* 1987r and *Enterococcus sp.* 1970g. The *E. hirae* 1981g wild-type isolate was only 0.25 mm and barely pink, making it more easily confused with non-enterococci. Like KF-Strep, MEA prevented the growth of the *A. viridans* and *L. lactis* isolates and inhibited the other potentially false positive, non-enterococci isolates. The *Pediococcus* isolate was punctiform and white. The MEA agar did provide some differentiation between species as *E. faecalis* isolates were red while the *E. faecium*, *E. hirae*, *E. avium* and *E. durans* isolates were pink.

2.3.1.1.3 Chromogenic media

The chromogenic medium, CEBA, performed satisfactorily for all the enterococci isolates, producing distinctive colonies with blue centres and lighter blue outer rings (Figure 2.1). The intensity of the blue colouration varied between isolates. Enterococci were 1-1.5 mm in diameter, with the exception of the *E. avium* ATCC 14025 reference culture and the *E. hirae* 1981g and *E. durans* 1998g wild type isolates, which were slightly smaller (<1 mm). Both the Gram-positive rod 1977 EV1 and Gram-positive coccus 1990 EV1 non-enterococci wild type isolates and the *Pediococcus* isolate were inhibited, as no growth was visible on the agar. The *A. viridans* reference strain and the
Figure 2.1 Chromogenic media preparations spread with enterococci: (a) CEBA
(b) CEBA 2
*L. lactis* isolate exhibited typical blue enterococci colony morphology, although the latter was only 0.5 mm in diameter.

2.3.1.4 Other media

The Mac2 medium produced various colour differences between isolates, although they were not consistent within species. Most of the enterococci colonies had red centres with either pink or transparent outer rings. The *E. gallinarum* ATCC 49573 reference strain and the *E. gallinarum* 1975V1, and *E. casseliflavus* 2003V1 wild type isolates had pink centres. Enterococci colonies were 0.5-1 mm in diameter, although the *E. avium* ATCC 14025 reference strain was smaller than this. The Mac2 was very inhibitory for all of the non-enterococci isolates except for false positive 1990b and the *A. viridans* reference strain. *A. viridans*, which exhibited typical enterococci morphology, was the same size as three of the *E. faecalis* cultures (1 mm) and larger than 14 of the other enterococci. False positive 1990b had pink centres similar to the *E. gallinarum*.

2.3.1.2 Recovery of enterococci

The recovery of the reference strains and the wild-type isolates on commercial enterococci media in relation to BHIA is illustrated in Figure 2.2 and counts between the media for each isolate were compared. Recovery on the selective media was not significantly different (*p* > 0.05) from the recovery on BHIA for all of the *E. faecalis* and *E. hirae* isolates, *E. casseliflavus* 1987r, *E. durans* 1998g, *E. faecium* ATCC 35667, *E. gallinarum* 1975V1 and *E. malodoratus* 1978r. The non-enterococci isolate, Gram-positive rod 1977 EV1 also recovered equally well on the selective media, although the colonies were white and punctiform on CEBA. Other isolates had significantly less recovery (*p* < 0.05) on specific media. Counts on ECA were significantly lower (*p* < 0.05) for *E. avium* ATCC 14025, *E. gallinarum* ATCC 49573 and *E. casseliflavus* 2003V1. Of the non-enterococci, false positive 1990b and *L. lactis* had significantly lower (*p* < 0.05) counts while *A. viridans* and *Pediococcus* did not grow at all. The *Pediococcus* isolate was originally isolated from ECA, however it may have been able to overcome the selectivity of the media due to the high density of the microbiota on the media at that time.
Figure 2.2 Recovery of enterococci and non-enterococci on commercial media for the:
(a) reference strains and (b) wild type isolates

LSD= Least Significant Difference, calculated separately for each isolate, p < 0.05
*E. avium* ATCC 14025 additionally had significantly lower \((p < 0.05)\) counts on BEA and Mac2, with the latter media also significantly lowering \((p < 0.05)\) the counts of *E. casseliflavus* 2003V1 and false positive 1990b, and preventing the growth of the *Enterococcus sp.* 1970g isolate. The ECA, Mac2, MEA and CEBA media each prevented the growth of two of the six non-enterococci isolates in various combinations and BEA prevented the growth of one non-enterococci isolate. The KF-Strep medium did not prevent the growth of any of the enterococci or non-enterococci isolates but did significantly decrease the counts of *A. viridans*.

Counts for all of the enterococci isolates on each media were averaged to determine the overall effect of the media across a range of species (Figure 2.3). Both ECA and Mac2 had significantly lower \((p < 0.05)\) counts compared with all of the other media. All of the other media recovered equivalent numbers of enterococci \((p > 0.05)\), with the exception of KF-Strep, which had a significantly higher \((p < 0.05)\) recovery compared to BEA.

### 2.3.2 Selection of commercial media for modification

From the colony morphology and isolate recovery results, CEBA appeared to have the most potential to recover enterococci selectively without hindering enterococci growth. The BEA, KF-Strep, MEA and CEBA media did not have significantly different \((p > 0.05)\) average enterococci recovery compared to the control media, BHIA, but *E. avium* ATCC 14025 did have significantly less growth on BEA. The potential for the esculin reaction to mask colony morphology on BEA was another negative feature of this media. The KF-Strep medium did not prevent the growth of any of the non-enterococci tested whereas MEA and CEBA both prevented the growth of two different non-enterococci isolates. While MEA prevented the growth of *A. viridans* and *L. lactis*, CEBA prevented the growth of the *Pediococcus* and Gram-positive coccus 1990 EV1 isolates. The TTC in MEA agar provided some differentiation between enterococci species, however MEA also inhibited eight of the enterococci isolates, as indicated by small colony size on the media, while only three enterococci isolates had a smaller colony size on CEBA. Enterococci, which were small on MEA, were also small on the other medium containing TTC, KF-Strep.
Figure 2.3 Recovery of all enterococci on various commercial media

LSD= Least Significant Difference, p < 0.05
A. viridans had significantly lower (p < 0.05) counts on KF-Strep. It was suspected that TTC could potentially be responsible for this inhibition, as well as for the decreased colony size of some enterococci isolates. To potentially improve the selectivity of CEBA and the differentiation of enterococci species, TTC was added to the formulation. Two levels of TTC (0.01 and 0.005 %) were assessed.

2.3.3 Assessment of modified media

2.3.3.1 Selection of modified media formulation

The addition of 0.01 and 0.005 % TTC to CEBA (CEBA 1, CEBA 2) decreased the size of the L. lactis isolate compared to CEBA. A. viridans was more noticeable at the lower TTC level (CEBA 2, 0.005 %) than on CEBA 1. Three of the enterococci isolates that were susceptible to the media containing TTC previously (E. gallinarum ATCC 49573, E. avium ATCC 14025, E. casseliflavus 1987r) were again inhibited slightly but performed better at the lower TTC level.

Reactions to the incorporation of SGPP were mixed. Colony size increased for three enterococci isolates at both TTC levels, for two isolates at 0.01 % TTC only (CEBA 3), and for four isolates at 0.005 % TTC only (CEBA 4). Seven enterococci isolates had no change in colony size at either TTC level. The incorporation of SGPP decreased the colony size of one enterococci isolate at both TTC levels and two enterococci isolates at 0.005 % TTC. Furthermore, SGPP only inhibited A. viridans at 0.005 % TTC but the other non-enterococci had no change in colony size.

Additional media formulations were made, reducing the TTC to 0.0025 % and 0.00125 %, to determine to what level the TTC could be reduced before non-enterococci would cease to be inhibited but the A. viridans strain began to increase in size. A brief experiment was performed where bile salts (1 and 2 %) were added to CEBA but this did not reduce the growth of A. viridans or L. lactis and obscured the blue colour reaction used to differentiate enterococci from other organisms. Therefore CEBA 2, with a TTC level of 0.005 %, was chosen for further assessment.
2.3.3.2 Colony morphology on modified media

The final media comparison was made between BHIA, MEA, CEBA, which was the most suitable commercial formulation and CEBA 2, which incorporated 0.005 % TTC. The addition of TTC aimed to provide the beneficial aspects of MEA by improving selectivity for enterococci and species differentiation. The red indicator (TTC) in the MEA combined with the blue chromogenic substrate (X-GLU) in the Chromocult Enterococci agar to create purple colonies, 0.5-1.5 mm, on CEBA 2 (Figure 2.1). The *Pediococcus* and Gram-positive coccus 1990 EV1 non-enterococci isolates did not grow on either CEBA or CEBA 2. Moreover, the non-enterococci isolate Gram-positive rod 1977 EV1, which grew on all of the previous commercial enterococci media, did not grow on CEBA 2. All of the enterococci isolates grew on MEA, CEBA and CEBA 2, but different morphologies were present.

The purple colour of the colonies on CEBA 2 was not always uniform throughout a culture. *E. faecium* ATCC 35667, false positive 1990b and *E. faecalis* 2000b all produced both purple and blue colonies, whereas *E. gallinarum* ATCC 49573 produced red and purple colonies. Additionally, *E. faecalis* ATCC 51299 had dark red, punctiform colonies and *E. avium* ATCC 14025 had purple colonies, which were only 0.5 mm. Two of the wild-type enterococci isolates (*E. hirae* 1981g and *E. durans* 1998g), which were inhibited on MEA, formed blue, 0.75 mm colonies on CEBA 2 media. Unfortunately, false positive 1990b, *A. viridans* and the *L. lactis* looked similar to these colonies. Although CEBA 2 improved the selectivity against the Gram-positive rod 1977 EV1, it did not eliminate all false positives. Furthermore it reduced the size and distinctiveness of some enterococci, increasing the risk of false negative results.

2.3.3.3 Differentiation of species

The MEA medium was best able to differentiate between enterococci species. *E. faecium* ATCC 35667, *E. hirae* ATCC 8043 and 1981g and *E. durans* 1998g had pink centres on this media while the *E. faecalis* cultures and other remaining isolates had red centres. Further differentiation to individual species was not possible. In mixed cultures, the reference strain *E. faecalis* ATCC 29212 could be distinguished from *E. faecium* ATCC 35667, *E. avium* ATCC 14025 and *E. hirae* ATCC 8043. *E. faecium* ATCC
35667 could be differentiated from *E. gallinarum* ATCC 49573, but the latter was inhibited on the media, producing colonies less than 0.25 mm in diameter. Distinctions could also be seen with the wild-type isolates. *E. faecalis* isolates (1967g, 1973r, 2000b) could be differentiated from *E. durans* 1998g, *E. gallinarum* 1975V1, *E. hirae* 1981g and *E. casseliflavus* 2003V1 isolates when in mixed culture.

Enterococci species could be partially differentiated on CEBA and CEBA 2, although differences in colour were minor. *E. gallinarum* ATCC 49573 was a greenish blue, compared to the blue colour of all the other isolates on CEBA. Most differences between isolates on CEBA were due to variations in colony size. Similarly with CEBA 2, most differences were due to minor variations in colony size. The *E. hirae* and *E. durans* wild type isolates were blue on CEBA 2, instead of the usual purple, but they were also partially inhibited, as colonies were smaller. In mixed culture, the *A. viridans* culture was smaller than the enterococci. Minor variations in the purple colour of enterococci colonies on CEBA 2 also occurred between and within media batches, which decreased the reliability of colour to indicate differences between isolates.

### 2.3.3.4 Recovery on modified media

The recovery for the majority of the enterococci on MEA, CEBA and CEBA 2 was not significantly different (*p* > 0.05) from the counts on BHIA (Figure 2.4). Counts on CEBA 2 were slightly lower (*p* < 0.05) for *E. faecalis* 1967g. A count was not performed for *E. casseliflavus* 2003V1, although it grew similarly to other enterococci, producing purple, 1 mm colonies.

Significant differences (*p* < 0.05) between the media occurred for the recovery of non-enterococci. *A. viridans* and *L. lactis* did not grow on MEA and the latter had significantly lower (*p* < 0.05) counts on CEBA 2. Three of the non-enterococci isolates,
Figure 2.4 Recovery of enterococci and non-enterococci on modified media for the: (a) reference strains and (b) wild type isolates

LSD = Least Significant Difference, calculated separately for each isolate, \( p < 0.05 \)
Gram-positive rod 1977 EV1, Gram-positive coccus 1990 EV1 and the *Pediococcus* isolate, did not grow on CEBA 2. Although 1977 EV1 had a colony count on CEBA for comparison with the other media, the colonies were less than 0.25 mm and white, and would not normally be counted since enterococci form blue colonies on this media. In effect, this culture would not have had a count on CEBA. Therefore, both CEBA and CEBA 2 effectively excluded three non-enterococci isolates each.

The CEBA medium was selected as the most suitable medium for the survey of enterococci in raw milk and dairy products and was the medium used for this purpose.

### 2.3.4 Prevalence in raw milk

A total of 211 raw milk samples were collected from six milk processing plants in three dairy regions in Victoria over a one year period, resulting in the isolation of 909 strains of enterococci. The data allowed analyses of the monthly incidence of enterococci at each factory (Figure 2.5), the range of counts seen across all of the factories (Figure 2.6) and variation in counts over four seasons across all of the factories (Figure 2.7). Enterococci were detected in 96% of the raw milk samples (detection limit $1 \log_{10} \text{cfu/mL}$), with counts ranging from $<1$ to $6.80 \log_{10} \text{cfu/mL}$ (Figure 2.5) with an average of $2.48 \log_{10} \text{cfu/mL}$ and most counts (77.3%) were less than $3 \log_{10} \text{cfu/mL}$. Factories A and F, which are located in the south of Victoria but in different regions, had the highest counts in individual samples (5 to $>6 \log_{10} \text{cfu/mL}$).

The average counts of enterococci in raw milk from each factory throughout the year were all between 2 and $3 \log_{10} \text{cfu/mL}$, however differences were found between factories ($p = 0.003$). Factory A had the highest enterococci counts ($p = 0.003$) compared to all of the other factories except Factory F. The average number of enterococci was not different ($p = 0.809$) between the three regions (north, east, and west of Melbourne, Victoria).
Figure 2.5 The monthly average raw milk enterococci counts for six dairy factories

SED = Standard error of difference of means
Figure 2.6 Range of enterococci counts obtained from raw milk

Figure 2.7 Counts of enterococci in raw milk over the four seasons averaged across all of the factories from April 2002 until February 2003

LSD = Least significant difference, p < 0.05
A distinct decrease in counts (p = 0.008) was observed in the winter season (particularly July-August samples for most factories, Figure 2.5) relative to the other seasons when considering the data across the three regions (Figure 2.7), with the winter counts 0.64 log$_{10}$ cfu/mL lower compared to those in summer.

### 2.3.5 Enterococci species in raw milk

A total of 909 isolates were confirmed as enterococci and analyses of species type in all of the raw milk samples determined which species were predominant (frequency of isolation), the mix of species at each factory (Figure 2.8a) and seasonal variation in species type (Figure 2.8b).

The following 13 species were detected in the 211 raw milk samples (% of total isolates): E. faecalis (74.3 %), E. faecium (8.6 %), E. hirae (8.5 %), E. casseliflavus (3.5 %), E. durans (2.9 %), E. asini (1.1 %), E. malodoratus (0.6 %) and E. gallinarum, E. mundtii, E. pseudoavium, E. raffinosus, E. saccharolyticus and E. sulfureus (0.1 %). Only one isolate each of the latter six species was found. The carbohydrate fermentation reactions of E. faecalis is shown in Figure 2.9.

E. faecalis, E. faecium, and E. hirae were the predominant species (defined as a species present in more than 50 % of the samples) in 72.9, 6.3 and 4.2 % of the raw milk samples with counts of ≥ 3 log$_{10}$ cfu/mL, respectively. The remaining eight samples (16.6 %) did not contain a predominant species. Four milk samples had enterococci counts of 5 log$_{10}$ cfu/mL or greater, with E. faecalis predominant and E. faecium predominant in only one sample.

The most prevalent Enterococcus species detected in each factory was E. faecalis (61.2 to 88.5 %) (Figure 2.8a). The greatest occurrence of E. faecalis was in Factory A while Factory F had the least. Conversely, Factory F had the most E. faecium while Factory A had the least. Notably, these two factories had different microbiota from the other factories and also yielded samples with the four highest counts.
Figure 2.8 Percentage of the 5 main enterococci species from raw milk: (a) at each factory averaged across all samples (b) in each season averaged across all factories
Figure 2.9 Carbohydrate fermentations of *E. faecalis* in Phenol Red broth
Seasonal differences for *E. faecalis* and *E. faecium* (Figure 2.8b) were observed, with the prevalence of *E. faecalis* increased in summer and *E. faecium* in autumn. *E. faecalis* remained the most prevalent species isolated in every season, including the peak milking season, spring (Dairy Australia, 2014). Seasonal distributions for *E. faecium* and *E. casseliflavus* were similar to each other, although numbers were lower for the latter species.

### 2.3.6 Prevalence in dairy products

#### 2.3.6.1 Pasteurised milk

Enterococci counts from pasteurised milk following manufacture at Factory B were below the limit of detection on spread plates (< 1 log$_{10}$ cfu/mL), and enterococci were only detected in five (20.8 %) of the 24 samples following enrichments using 100 mL of milk in 2XCEB. Since the limit of detection in the 100 mL enrichments was 0.01 cfu/mL, assuming a minimum number in the 100 mL volume this represented a calculated decrease in the average raw milk enterococci count (2.48 log$_{10}$ cfu/mL) of approximately 4 log$_{10}$ cfu/mL. Three of the five positive samples were obtained in the autumn and one each in winter and summer, while none were detected in spring. The highest raw milk counts in samples from Factory B were 3 to 4 log$_{10}$ cfu/mL in spring (p = 0.027), so that persistence after pasteurisation did not correlate with higher initial bacterial loading in raw milk.

#### 2.3.6.2 Butter

Sourced from Factories G and H, five butter samples contained enterococci only in the 100 g sample size, while a further nine samples also had enterococci in 10 g of butter (Table 2.5). Enterococci were only detected on CEBA and not on the plates containing vancomycin, VCEBA. Of the 16 butter samples tested, only two of the samples were not found to contain enterococci in 100 g. Both of these samples were from Factory G in spring. Enterococci were detected in 10 g of the other two spring samples, from Factory H. All four of the summer butter samples contained enterococci in 10 g. In the autumn, enterococci were detected in 10 g of two butter samples but only in 100 g of the other two samples. Similarly in winter, enterococci were detected in 10 g in one sample but
only in 100 g of the other three samples. The most samples with lower levels of enterococci (in 100 g but not 10 g) occurred in winter, which was when the least amount of enterococci was detected in the raw milk. The presence or absence of salt did not appear to affect the level at which enterococci were detected in the butter (Table 2.6).

2.3.6.3 Cheddar cheese
All of the Cheddar cheese samples came from Factory M. Enterococci were found in seven out of the eight Cheddar cheese samples tested at 100 g (Table 2.5). No enterococci were detected in 100 g in one of the two autumn samples. The two spring samples also contained enterococci in 10 g of Cheddar cheese. No enterococci were detected on the VCEBA plates.

2.3.6.4 Milk powder
Fourteen milk powder samples were tested. Enterococci were detected in 100 g in nine of the 14 samples (Table 2.5). All of the autumn samples from Factories J and K also contained enterococci in 10 g. The level of enterococci detected in the one full cream milk powder sample from Factory K was not different from the other three low heat skim milk powders from Factories J and K in the autumn. In the winter, enterococci were not detected in 100 g of the two milk powder samples from Factory J but were in 100 g of milk powder from Factory L. Two samples from Factories J and L in the spring contained enterococci in 100 g of milk powder. The other sample from Factory J did not contain enterococci in 100 g. Enterococci were detected in 10 g of the milk powder samples from Factory J but not in 100 g of the samples from Factory L in the summer. The milk powders with the highest levels of detection of enterococci occurred in autumn and summer, when raw milk enterococci numbers were greatest. None of the samples had enterococci on VCEBA.

2.3.7 Enterococci species in dairy products
Six species of enterococci were found in the pasteurised milk, butter, Cheddar cheese and milk powder samples (Figure 2.10). They were *E. faecalis*, *E. faecium*, *E. hirae*, *E. durans*, *E. casseliflavus* and *E. malodoratus*. *E. durans* was detected at the highest
Table 2.5 Level at which enterococci were detected in long-life dairy products

<table>
<thead>
<tr>
<th>Product</th>
<th>Enterococci detected in 10 g</th>
<th>Enterococci detected in 100 g</th>
<th>Enterococci not detected in 100 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butter (n = 16)</td>
<td>56.3 %</td>
<td>87.5 %</td>
<td>12.5 %</td>
</tr>
<tr>
<td>Cheddar cheese (n = 8)</td>
<td>25.0 %</td>
<td>87.5 %</td>
<td>12.5 %</td>
</tr>
<tr>
<td>Milk powder (n = 14)</td>
<td>42.9 %</td>
<td>64.3 %</td>
<td>35.7 %</td>
</tr>
</tbody>
</table>

Table 2.6 Level at which enterococci were detected in salted and unsalted butter

<table>
<thead>
<tr>
<th>Factory</th>
<th>Level of enterococci detected in each season</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Autumn</td>
</tr>
<tr>
<td>G</td>
<td>10 g</td>
</tr>
<tr>
<td>H</td>
<td>100 g</td>
</tr>
</tbody>
</table>

Shaded cells indicate unsalted butter samples. All other samples were salted.

Figure 2.10 Percentage of Enterococcus species detected in each of the milk products
frequency among pasteurised milk samples (4/5) in the summer and autumn; the remaining positive sample contained *E. malodoratus* (1/5) in the winter, noting that these species were not predominant in raw milk samples from this factory (Figure 2.8a). Butter contained *E. faecalis* in 12 of the 16 samples, with *E. faecium* and *E. casseliflavus* also being detected in three and two samples, respectively. The Cheddar cheese samples had the greatest species diversity, with five species detected. The species found in the greatest number of Cheddar cheese samples was *E. malodoratus*. *E. faecalis*, *E. faecium* and *E. hirae* were detected in one sample each of this product. The only species isolated from the milk powder was *E. faecium*, in 100% of the samples in which enterococci were detected.

Given that pasteurised milk and Cheddar cheese samples came from only one factory source, and that milk powder samples contained the one species of enterococci, the only product type that allowed comparisons of species types across multiple factories was butter. *E. faecalis* was isolated from the butter samples of both Factories G and H. *E. faecium* was only detected in Factory G butter samples and *E. casseliflavus* was detected in butter samples from Factory H.

Determination of the seasonal variation in the enterococci species in the milk products was also limited. Enterococci were only detected in one fifth of the pasteurised milk samples. Three of the five pasteurised milk samples were obtained in the autumn, of which all three samples contained *E. durans*. One pasteurised milk sample in winter and one sample in summer contained *E. malodoratus* and *E. durans*, respectively. In the butter samples, *E. faecalis* was isolated all year round, both *E. faecium* and *E. casseliflavus* were obtained in autumn and *E. faecium* was also isolated in winter. In the Cheddar cheese, *E. malodoratus* was isolated in spring, summer and autumn. *E. durans* and *E. faecium* were isolated from the Cheddar cheese in the summer, *E. durans* and *E. hirae* were isolated in the winter and *E. faecalis* was isolated in the spring.
2.4 Discussion

2.4.1 Preliminary media assessment

The use of bile and esculin was first described in 1926 by Meyer and Schonfeld (Devriese and Pot, 1995). Although BEA is used in water testing (Standards Australia, 1995a,b) and ECA had been used in hospital screening of enterococci, the use of media containing esculin has disadvantages. If an agar plate has a high concentration of bacteria that hydrolyse esculin, the entire plate can be blackened. This certainly happened with the enterococci. Further complicating the identification of enterococci was that colony morphology varied between isolates, which made enterococci difficult to recognise on blackened plates, as the media was not completely selective for enterococci. In addition, recovery on one of the two media with bile and esculin, ECA, was less than on the control agar (BHIA) and on four of the other enterococci media assessed (BEA, KF-Strep, MEA, CEBA).

From the group of media containing TTC, MEA prevented the growth of *A. viridans* and *L. lactis*. It also decreased the colony size of the other three non-enterococci tested. However, it decreased the size of eight of the enterococci isolates as well. This was unfortunate since MEA provided the best differentiation between *Enterococcus* species groups. At the time that the work was commenced, the species groups were the *E. faecium* group, which included *E. faecium*, *E. hirae*, *E. durans* and *E. mundtii*, the *E. avium* group consisting of *E. avium*, *E. pseudoavium*, *E. malodoratus* and *E. raffinosus*, the *E. gallinarum* group, consisting of *E. gallinarum*, *E. casseliflavus* and *E. casseliflavus*, the *E. cecorum* group, consisting of *E. cecorum* and *E. columbae*, and lastly, *E. faecalis*, which was on its own (Devriese and Pot, 1995). The *E. faecium* group does not reduce TTC, producing pink colonies on this type of media, whereas *E. faecalis*, and other enterococci species that reduce TTC, produce red colonies on MEA (Devriese and Pot, 1995). The other medium with sodium azide and TTC, KF-Strep, was not a useful medium since it did not prevent the growth of any of the non-enterococci, which appeared similar to the slightly inhibited enterococci isolates with smaller colony sizes.
The enterococci generally did not grow well on Mac2, which contained bile salts, neutral red and crystal violet, as the colonies produced were smaller than on the other selective media. Although the media inhibited the growth of three of the non-enterococci isolates, colony size of the *A. viridans* isolates was the same as three of the *E. faecalis* isolates and larger than the majority of the other enterococci isolates. The Mac2 medium also recovered significantly less enterococci compared with the control media and was thus unsuitable.

All of the enterococci isolates grew satisfactorily on CEBA, which recovered similar numbers of enterococci as the control media. Two of the non-enterococci isolates, *A. viridans* and *L. lactis*, exhibited typical enterococci colony morphology on CEBA, although the latter was only 0.5 mm in diameter. The CEBA medium was selected as the most suitable commercial medium for further study as it did not inhibit enterococci (as did ECA, Mac2 and MEA), it did not have the esculin problems of ECA and BEA and it inhibited non-enterococci better than KF-Strep.

Since MEA and CEBA both contained sodium azide, and enterococci were not inhibited on CEBA, it was suspected that TTC, one of the agents in MEA, potentially inhibited some enterococci. It was also potentially inhibiting the *A. viridans* and *L. lactis* cultures. The TTC is known to inhibit Gram-positive bacteria (Weinberg, 1953), depending on the concentration used. Therefore TTC was added to CEBA, which was selected as being the commercial medium most suitable for adjustment. The TTC is present in the KF-Strep and MEA media formulations at 0.01 %. It was tested at two levels initially, 0.01 and 0.005 %. The addition of 1 % SGPP was also assessed, as it is used in the Amyl KF *Streptococcus* formulation to contribute high energy phosphate which according to the manufacturer is used in enzymatic reactions necessary for biological metabolism (Amyl Media, 2000). The addition of SGPP stimulated the growth of some enterococci isolates but inhibited the growth of other isolates. It was therefore not beneficial.

The CEBA 2 medium, containing 0.005 % TTC, was selected for final assessment, since the higher level of TTC inhibited some enterococci species and the lower levels briefly
appraised were not beneficial as the media no longer inhibited *A. viridans*, one of the main targets of this work. The recovery of enterococci on CEBA 2 was not different to the recovery on the control media, MEA or CEBA. The *Pediococcus*, Gram-positive rod 1977 EV1 and Gram-positive coccus 1990 EV1 wild type isolates were discounted on both CEBA and CEBA 2, although *L. lactis* was only reduced in number on CEBA 2 and *A. viridans* grew equally well on both. Furthermore, colony colour on CEBA 2 was not consistent, possibly because the TTC did not mix well with the chromogenic substrate (X-GLU). This made the addition of TTC to CEBA not worthwhile to pursue.

### 2.4.2 Survey of raw milk and dairy products

Enterococci are commonly found in raw milk and processed products, with different microbiota reported in different countries, reflecting local practices and levels of hygiene (Batish and Ranganathan, 1984b; Wessels et al., 1988; Citak et al., 2005). It is generally accepted that farms and manufacturing facilities may develop a specific microbiota, giving rise to typical product traits which make these distinctive or regionally recognisable (Giraffa, 2002; Quigley et al., 2011). Sources tracking of entry of enterococci into raw milk and subsequent transmission into processed products has indicated persistence of particular species of strain types, where the likely source of reintroduction of these is through milk harvesting and processing equipment as well as through common microbiota residing in workers involved in the supply chain (Gelsomino et al., 2001, 2002), although it is not clear whether enterococci species originated in cows or humans and are subsequently shared microbiota (Gelsomino et al., 2002; Kagkli et al., 2007). Consequently, at the outset of the survey, it was anticipated that the enterococcal microbiota may vary between dairying regions, and over time, in Victoria due to influences including climate and seasonal temperature differences, water supply, the specific microbiota in farms and processing plants servicing the regions and the transport systems operating, given that prior studies have indicated the diversity of enterococci found in particular farming and manufacturing settings (Cogan et al., 1997; Franciosi et al., 2009; Jackson et al., 2010). The centres of the regions in the current study are approximately 250 km apart from each other, with milk collection within the region so may represent potentially distinct pools of microbes that have evolved in the
particular supply systems around the processing plants. Transport from farms involves varying distances so the critical point for determining raw milk quality is in bulk storage prior to processing, which provided an overview of microbial quality in terms of enterococci count. The data collected over this one year survey indicated that there were significant differences between individual factories. However, factory location in the state did not correlate with differences in the incidence, species or counts despite differences in climatic conditions between the regions. Temperature differences in summer were not an important factor, which implies good practice in milk collection, transport and compliance to regulated temperature control requirements. Milk must be chilled to $\leq 5 \, ^\circ C$ within 3.5 h of commencement of milking and transported at $\leq 5 \, ^\circ C$: it is the manufacturer’s responsibility to ensure validation of temperature control procedures and equivalence demonstrated to ensure minimization of pathogenic organism growth (Dairy Food Safety Victoria, 2015). Significant differences in load and species type were observed between factories, suggesting that hygiene or management practices in supply and storage needs addressing and this was more important than regionality. Distinctive enterococci microbiota were observed between the factories but \textit{E. faecalis} dominated in all factories. \textit{E. faecium} was also found in all of the factories, and was more distinctive in one factory (Factory F) which had a decreased presence of \textit{E. faecalis}.

Seasonality was observed in the raw milk when data from all samples taken from all factories was pooled. Lower counts were obtained in winter and the ratio of species mixes also differed in every season. \textit{E. faecium} was generally proportionately higher when the incidence of \textit{E. faecalis} was lower although \textit{E. faecalis} dominated all samples. This was similar to the dominance of this species in other reports (Batish and Ranganathan, 1984b; Schlegelova et al., 2002; Citak et al., 2005).

There was a low prevalence of the more thermoduric species in the raw milk (\textit{E. faecium}, \textit{E. hirae} and \textit{E. durans}). Survival of a few enterococci occurred at a detection level of 0.01-0.09 log$_{10}$ cfu/ml after pasteurisation (72 $^\circ$C, 15 s), with \textit{E. durans} being most prevalent. \textit{E. durans} was one of the more thermoduric species assessed in pasteurised milk, whereas \textit{E. faecalis} was comparatively more heat sensitive (Chapter 3).
Enterococci numbers were much lower in the milk products compared to the raw milk, and the products required enrichment in order to detect the enterococci. An incubation time of 72 h was used to allow additional time for the resuscitation of damaged cells in the large sample volume. Even with enrichment, enterococci were not detected in all of the butter, Cheddar cheese and milk powder samples despite the presence of enterococci in most raw milk samples. Lower enterococci counts in the dairy products compared to the raw milk indicate that some of the enterococci population has been reduced by the processing procedures, which entail milk pasteurisation treatments at minimum. Furthermore, the range of enterococci species found in the butter, Cheddar cheese and milk powder corresponded with those that were detected in the pasteurised milk samples. No seasonal effect was apparent in the detection of enterococci in butter and milk powder. Enterococci were detected in a smaller quantity of Cheddar cheese in the spring compared to the other seasons, but conclusions are hard to draw from this considering that there were only two cheese samples tested in each season.

Enrichment strategies for detecting very low numbers of survivors, or damaged cells, were useful albeit semi-quantitative. This allowed the order of magnitude of contaminants to be determined and also facilitated detection and identification of the thermoduric species surviving pasteurisation. It enabled the detection of the range of species that survived pasteurisation which would have been otherwise undetected by standard plating methods. Survival of *Enterococcus* species in pasteurised milk is important in terms of the ability to grow subsequently and contribute to spoilage or pose health risks: this study showed that it is important to use enrichment methods to detect and identify the species which survive in low numbers.

2.5 Conclusion

Six commercial enterococci media, Enterococcosel Agar, Bile Esculin Agar, *m Enterococcus* Agar, KF *Streptococcus* Agar, Chromocult Enterococci media and MacConkey Agar No. 2, and a modified media formulation, Chromocult 2, were assessed
for morphology and recovery of enterococci and other related bacteria. None of the media were able to exclusively select for enterococci, however Chromocult medium was less inhibitory to the enterococci than the other media allowing good recovery of all eight species tested. Differentiation of species by colony morphology on Chromocult medium was not possible. Therefore species needed to be distinguished using further biochemical tests. As Chromocult medium did not prevent the growth of all non-enterococci, confirmation tests were required to distinguish enterococci from other species, such as aerococci and lactococci, which may occur in milk and dairy products. This was the medium selected for the isolation and enumeration of enterococci in the survey of raw milk and dairy products.

The survey of enterococci in raw milk from factory silos demonstrated that these bacteria are widespread in milk prior to manufacture. The occasional occurrence of high levels of enterococci in a small number of samples suggests that environmental contamination is at times high and hygiene of milk collection or in factories needs to be improved in some instances. Seasonal differences were observed with lower counts occurring in winter. This has not been reported in other studies of bovine raw milk internationally. \textit{E. durans} was detected in the greatest number of pasteurised milk samples after manufacture although \textit{E. faecalis} was the predominant species detected in the raw milk. The level of detection of enterococci in pasteurised milk was low and did not coincide with higher raw milk counts, indicating that the main enterococci population was heat-sensitive.
CHAPTER 3 - HEAT RESISTANCE OF THERMODURIC ENTEROCOCCI ISOLATED FROM MILK
3.1 Introduction

Pasteurisation of milk is applied primarily to eliminate the presence of pathogenic microorganisms although it will also reduce the levels of spoilage bacteria in raw milk (Holsinger et al., 1997; Lewis, 2003). A typical high temperature-short time (HTST) pasteurisation treatment of raw milk involves rapidly heating the milk to a minimum of 72 °C for 15 s, followed by rapid cooling to 4.5 °C (Lewis, 2003; FSANZ, 2004). Pathogens such as *Campylobacter* and *Salmonella* can undergo a 5-8 log \(_{10}\) reduction with such a treatment (Lewis, 2003). Throughout history, pasteurisation conditions have been modified as new information about the heat resistance of target microorganisms has been identified (Holsinger et al., 1997), and it is important that legislative requirements and food processors keep up with new information as it becomes known.

Enterococci may enter raw milk and dairy products during manufacture from human, animal or environmental sources and have been suggested as useful indicator organisms for process hygiene (Garg and Mital, 1991; Giraffa, 2003). Several authors have alluded to the heat resistance of enterococci (Franz et al., 1999; Giraffa, 2002; Martinez et al., 2003) and it has been noted that enterococci can survive pasteurisation temperatures (Giraffa et al., 1997; Rao et al., 1986) but the extent of their survival is unclear. Although enterococci are potentially beneficial in some dairy products as starter or probiotic cultures (Giraffa, 2003), they are also considered to be potential spoilage microorganisms and may present a health risk to humans through the production of biogenic amines during cheese manufacture (Garg and Mital, 1991). Some species are opportunistic pathogens which may carry intrinsic or acquired antibiotic resistance (Franz et al., 1999). The presence of these organisms in food is undesirable because of the risk of transferring resistance to other pathogens and it is generally undesirable to have such microorganisms in food. The purpose of this work was to determine the effect of pasteurisation conditions on the survival of heat resistant enterococci and to obtain data that would allow the prediction of the thermal treatments required to inactivate populations of thermoduric enterococci which may be found in raw milk.
3.2 Materials and Methods

3.2.1 Strains and culture maintenance

Raw milk was collected monthly from the refrigerated bulk milk silos at six dairy factories (A, B, C, D, E and F) in Victoria, Australia, over one year (Chapter 2.2.5.1). Laboratory pasteurisation of 100 mL of 71 raw milk samples was performed by submerging bottles in a waterbath at 63 °C for 30 min, followed by rapid cooling in ice water. A control bottle containing a thermocouple in UHT milk was used to monitor temperature throughout the heat treatment. The pasteurised milk was spread (0.1 mL) onto CEBA (Chapter 2.2.3) in duplicate (Standards Australia, 1991, AS1766.1.4) and incubated at 37 °C for 48 h. Surviving enterococci were selected from colonies on CEBA. The identification of the resulting 176 isolates was confirmed using biochemical tests (catalase negative, PYR and growth in 6.5 % NaCl and at 45 °C) (Chapter 2.2.6.5). Isolates were speciated according to the method of Manero and Blanch (1999) (Chapter 2.2.6.6). One isolate each of the four predominating species was chosen from each factory and season for further analysis, selecting from all three milk samples in a season, where possible. This provided a total of 61 isolates consisting of 11 \( E. faecalis \), 22 \( E. faecium \), 19 \( E. durans \) and 9 \( E. hirae \) isolates. The \( E. faecalis \) ATCC 19433 Type strain (reported as NCTC 775) has been used to validate thermal inactivation processes (Bradley and Fraise, 1996). It was used as a control in this study (Chapter 2.2.1) for comparative purposes in the immersed coil procedure.

Enterococci isolates were grown in BHIB at 37 °C for 18-24 h and inoculated onto BHIA slopes (Chapter 2.2.2). Working cultures were obtained from the agar slopes and were stored long-term as indicated in Chapter 2.2.2.
3.2.2 Thermotolerance ranking

The 61 isolates were grown for 20 h in BHIB, diluted to $10^7$ cfu/mL in 10 mL of BHIB and pasteurised in the laboratory. The pre- and post-pasteurisation samples were spiral plated (Don Whitley Scientific Limited, Shipley, West Yorkshire, England), in duplicate, onto BHIA plates. Post-pasteurisation samples were also pour plated in duplicate (1 mL, in BHIA) (Standards Australia, 1991, AS1766.1.3). The plates were incubated at 37 °C for 72 h and the colonies were counted. Log$_{10}$ reductions were calculated by subtracting the log$_{10}$ cfu/mL post-pasteurisation count from the log$_{10}$ cfu/mL pre-pasteurisation count. The results were used to rank the isolates from each species into the order of highest to lowest log$_{10}$ reduction after pasteurisation. For each of the four species, one isolate with the median heat resistance and one isolate with the greatest heat resistance were selected to represent typical and more highly heat resistant enterococci, respectively.

3.2.3 Thermal death determinations

3.2.3.1 Immersed coil procedure

The eight selected pasteurised milk isolates (Table 3.1) and *E. faecalis* ATCC 19433 were tested for their survival at combinations of 63, 66, 69, 72, 75, 78 and 81 °C, depending on heat sensitivity, using an immersed coil apparatus (Sherwood Instruments, Lynnfield MA, USA) (Figure 3.1). The isolates were inoculated individually into 10 mL of BHIB and incubated at 37 °C for 20 h. The concentration of cells was adjusted to between $10^7$ and $10^8$ cfu/mL with BHIB and 10 mL of the diluted culture were injected into the immersed coil using a sterile syringe (Terumo, Binan, Laguna, Philippines). The coil instantaneously heated the culture to the required temperature and discharged 400 µL at ten equidistant time periods into 3.6 mL of chilled 0.1 % Bacteriological Peptone. Samples were immediately placed in ice water, ensuring rapid cooling. The samples, before and after heating, were serial diluted as necessary. Plates were incubated at 37 °C for 72 h before counting.
Table 3.1 Enterococci isolates used in thermal death determinations

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Species</th>
<th>Factory</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 19433</td>
<td><em>E. faecalis</em></td>
<td>N/A</td>
</tr>
<tr>
<td>2350p1</td>
<td><em>E. faecalis</em></td>
<td>A</td>
</tr>
<tr>
<td>2356p1</td>
<td><em>E. faecalis</em></td>
<td>F</td>
</tr>
<tr>
<td>2276p1</td>
<td><em>E. faecium</em></td>
<td>C</td>
</tr>
<tr>
<td>2299p2</td>
<td><em>E. faecium</em></td>
<td>F</td>
</tr>
<tr>
<td>2128p1</td>
<td><em>E. durans</em></td>
<td>D</td>
</tr>
<tr>
<td>2151p1</td>
<td><em>E. durans</em></td>
<td>B</td>
</tr>
<tr>
<td>2239p2</td>
<td><em>E. hirae</em></td>
<td>A</td>
</tr>
<tr>
<td>2452p2</td>
<td><em>E. hirae</em></td>
<td>E</td>
</tr>
</tbody>
</table>

Figure 3.1 Immersed coil apparatus
3.2.3.2 Optimum incubation time

The optimum incubation period for recovering surviving, possibly heat damaged, cells on plates was determined by measuring the recovery of *E. durans* 2151p1 and *E. faecalis* 2350p1 after heat treatment at 75 °C. After 75 °C heat treatment, the plates for these two isolates were incubated at 37 °C for 24, 48, 72 and 96 h. The counts were analysed using Analysis of Variance (Chapter 3.2.3.3) to determine the optimum incubation time after which additional incubation did not result in the detection of further colonies.

3.2.3.3 Calculations

Thermotolerance was determined once for all isolates by calculating the reduction in viability, in log_{10} cfu/mL, after laboratory pasteurisation. Standard deviations were calculated for each species (Microsoft® Excel 2003). For the thermal death determinations using the immersed coil, survivors of each isolate (log_{10} cfu/mL) were plotted against time using single determinations at each temperature, generating scatter graphs using Microsoft Excel. The D values (time required for counts to decrease by one log_{10} cfu/mL) were calculated from the resulting linear regression equations on the linear portion of the curves. Two thermal death curves were generated for all of the isolates, except 2356p1, at the key temperature of 72 °C. Statistical differences between the D values for two independent experiments were analysed using Analysis of Variance. Analysis of Variance was conducted using GenStat Release 13.1 (VSN International Ltd., Hemel Hempstead, England). The log_{10} D values were plotted against the temperature at which the D value was determined for four temperatures (selected from 63, 66, 69, 72, 75 and 78 °C) on X-Y scatter graphs. Linear regressions were performed for each isolate to calculate the z values (decrease in D value by one log_{10} unit).

The Weibull model was originally developed for application to non-linear thermal inactivation data (Peleg and Cole, 1998). In this study, the model (LogS = -b*t^n) was used to calculate the log_{10} reductions at 72 °C for 15 s, where LogS is the log_{10} reduction in the population of microorganisms, t is the time, and b and n are constants for the scale and shape parameters, respectively (Mattick et al., 2001). Additionally, this model was used to calculate the time required to achieve a six log_{10} reduction at 72 °C.
3.3 Results

3.3.1 Thermotolerance ranking

Between nine and 12 enterococci isolates were tested from each of six factories for their relative ability to survive laboratory pasteurisation. *E. faecalis, E. faecium, E. durans* and *E. hirae* were represented in all of the factories, except Factory D, in which *E. faecalis* was not isolated from the laboratory pasteurised milk. The range of $\log_{10}$ reductions achieved by laboratory pasteurisation for each species were 3.5 to 0.3 $\log_{10}$ cfu/mL for *E. faecalis* (Figure 3.2a), 3.2 to 0.1 $\log_{10}$ cfu/mL for *E. faecium* (Figure 3.2b), 2.7 to 0.4 $\log_{10}$ cfu/mL for *E. durans* (Figure 3.2c) and 4.0 to 0.4 $\log_{10}$ cfu/mL for *E. hirae* (Figure 3.2d). Different scales were shown for the X axes of these figures to account for the different number of isolates obtained for each species due to the relative heat sensitivities of the different species. The average $\log_{10}$ reduction for all of the isolates was 1.1 $\log_{10}$ cfu/mL. Eleven isolates of *E. faecalis* had an average $\log_{10}$ reduction of 1.3 $\log_{10}$ cfu/mL, 22 isolates of *E. faecium* had an average $\log_{10}$ reduction of 1.0 $\log_{10}$ cfu/mL and 19 isolates of *E. durans* had an average $\log_{10}$ reduction of 0.9 $\log_{10}$ cfu/mL. *E. hirae* was the most sensitive to laboratory pasteurisation with nine isolates having an average $\log_{10}$ reduction of 1.5 $\log_{10}$ cfu/mL. Four of the six most heat resistant *E. faecium* isolates were all from the same location, Factory C. In contrast, five of the seven most heat sensitive isolates from all of the species were from Factories A and D.

From these results, one isolate with greater heat resistance and one isolate with median heat resistance were selected for each species to evaluate D and $z$ values. The selected isolates were *E. faecalis* 2350p1 and 2356p1, *E. faecium* 2276p1 and 2299p2, *E. durans* 2128p1 and 2151p1 and *E. hirae* 2239p2 and 2452p2.
Figure 3.2 Distribution of log_{10} reductions after laboratory pasteurisation (63 °C/30 min) of: (a) *E. faecalis* (b) *E. faecium* (c) *E. durans* (d) *E. hirae*

Factory A □ B ■ C □ D ■ E □ F ■
3.3.2 Thermal death determinations

3.3.2.1 Incubation time

To determine the incubation time required to recover heat stressed or damaged cells, two isolates were evaluated in the immersed coil apparatus at 75 °C for up to 5.25 min. *E. durans* 2151p1 and *E. faecalis* 2350p1 colonies were counted after 24, 48, 72 and 96 h of incubation at 37 °C. Two-way Analysis of Variance for *E. durans* 2151p1 indicated that an incubation of 24 h yielded significantly lower (p < 0.001) counts compared to the 48, 72 and 96 h incubation times. The counts obtained at 72 h were not significantly different (p > 0.05) to the 48 h counts, however the 48 h counts were significantly lower (p < 0.001) than the 96 h counts. The interaction between incubation time and holding period was not significant for *E. durans* 2151p1 (p = 0.718) but it was for *E. faecalis* 2350p1 (p < 0.001) (Table 3.2). Increased incubation did not change the counts for the *E. faecalis* 2350p1 unheated control sample (holding period of 0 s). The counts were significantly higher (p < 0.001) after 48 h incubation compared with 24 h incubation after the first 10 s holding period. When the holding period was extended beyond 10 s, the counts were significantly higher (p < 0.001) only after an incubation time of 72 h. Further recovery was not significantly increased (p > 0.05) with incubation to 96 h. These results indicate that incubation of the plates for 72 h is required for the recovery of heat stressed or damaged cells at 75 °C. All plates for the remaining heat treatments were incubated for a period of 72 h.
Table 3.2 Recovery of *E. faecalis* 2350p1 after heat treatment at 75 °C for the determination of optimum incubation time

<table>
<thead>
<tr>
<th>Holding period at 75 °C</th>
<th>Counts (log₁₀ cfu/mL) after increasing incubation times</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>0 s</td>
<td>7.86&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 s</td>
<td>5.57&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>20 s</td>
<td>5.67&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>30 s</td>
<td>5.66&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>40 s</td>
<td>5.52&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>50 s</td>
<td>5.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>60 s</td>
<td>3.64&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>70 s</td>
<td>3.78&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>80 s</td>
<td>4.54&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>90 s</td>
<td>4.18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>100 s</td>
<td>4.16&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a, b, c</sup> Different letters indicate significant differences (p < 0.001) within a row, lsd = 0.2394
3.3.2.2 Survival curves

The prepared cultures were each heated at five temperatures between 63 and 81 °C and samples were taken for up to 10 time periods, not including the unheated control. Time periods varied depending on the heating temperature, with intervals of 5 to 10 min at the lowest temperature and 7 to 10 s at the highest temperature. Survival curves were constructed to assist in the calculation of D values for each isolate. The survival curves at 63 °C had an average reduction of approximately $0.5 \log_{10} \text{cfu/mL}$ after 30 min for all of the pasteurised milk isolates (Figure 3.3a). This was markedly different from the *E. faecalis* ATCC 19433 Type strain, which had a $3.8 \log_{10} \text{cfu/mL}$ decrease in this time. *E. faecalis* 2350p1 and *E. faecium* 2276p1 had 1.9 and $2.1 \log_{10} \text{cfu/mL}$ reductions, respectively, after 100 min, whereas *E. hirae* 2452p2 and *E. faecalis* 2356p1 incurred the greatest reductions which were $3.9$ and $4.4 \log_{10} \text{cfu/mL}$, respectively. At 72 °C, *E. faecalis* ATCC 19433 was again the least heat resistant (Figure 3.3b), sustaining a $3.5 \log_{10} \text{cfu/mL}$ decrease after 15 s. With the exception of one other *E. faecalis* isolate (2356p1), the majority of the pasteurised milk isolates did not show a similar reduction in counts until after five minutes at 72 °C.

The higher temperatures of 75 °C (Figure 3.3c), 78 °C (Figure 3.3d) and 81 °C were assessed for six of the isolates (2128p1, 2151p1, 2276p1, 2299p2, 2350p1, 2452p2) which showed greater thermal resistance. All of the pasteurised milk isolates were viable after one minute at 75 °C, with reductions ranging from $5.0 \log_{10} \text{cfu/mL}$ for the most sensitive isolate (*E. faecalis* 2356p1) to $0.8 \log_{10} \text{cfu/mL}$ for the most heat resistant isolate (*E. hirae* 2452p2). At 78 °C, the two most heat sensitive pasteurised milk isolates were not detectable past the first time point of 10 s, while the remaining six pasteurised milk isolates had reductions ranging from $0.8 \log_{10} \text{cfu/mL}$ (*E. faecalis* 2350p1) to $2.9 \log_{10} \text{cfu/mL}$ (*E. faecium* 2276p1) after 10 s. Notably, these isolates were more similar after 100 s with reductions of 4.3 and $4.9 \log_{10} \text{cfu/mL}$, respectively. None of these isolates had detectable counts after 10 s at 81 °C (detection limit of $10^2 \text{cfu/mL}$). For the two most heat sensitive isolates, *E. faecalis* 2356p1 and *E. hirae* 2239p2, survival was additionally assessed at 69 °C to provide a fourth temperature with which to generate D values, since the generation of a survival curve was not possible at 78 °C.
Figure 3.3 Survival of nine enterococci isolates at: (a) 63 °C (b) 72 °C (c) 75 °C (d) 78 °C using the immersed heating coil. The time scale varies between the temperatures to illustrate the characteristics of the curves.

- ATCC 19433 *E. faecalis*
- 2350p1 *E. faecalis*
- 2356p1 *E. faecalis*
- 2276p1 *E. faecium*
- 2299p2 *E. faecium*
- 2128p1 *E. durans*
- 2151p1 *E. durans*
- 2239p2 *E. hirae*
- 2452p2 *E. hirae*
As *E. faecalis* ATCC 19433 was also rapidly killed at 75 °C, it was assessed at 63, 66, 69 and 72 °C. At 69 °C, *E. faecalis* 2356p1 and *E. hirae* 2239p2 incurred reductions of 4.3 and 0.5 log_{10} cfu/mL after five minutes, but *E. faecalis* ATCC 19433 had already been reduced by 4.1 log_{10} cfu/mL after 2.5 min.

3.3.2.3 D values
The D values decreased with increases in temperature as expected for all of the isolates, but the rate of decrease was different among isolates. For example, although *E. faecalis* 2350p1 had a much higher D values at 63 °C than all of the other isolates tested (Table 3.3), at 78 °C it was almost the same as the other isolates, which all had D values of 0.4 or 0.5 min. *E. hirae* 2452p2 and *E. hirae* 2239p2 had comparable D values at 63 °C but their calculated heat resistance at the higher temperatures was markedly different. While *E. hirae* 2239p2 could not be detected after the first time point at 78 °C and was only tested at a maximum of 75 °C, *E. hirae* 2452p2 could be assessed at 78 °C. Heat resistance at lower temperatures was not necessarily an indication of resistance at the higher temperatures. However, the Type strain *E. faecalis* ATCC 19433, which had the lowest D value at 63 °C, also proved to be less tolerant than any of the other isolates at the higher temperatures. It had a D value of 0.5 min at the additional temperature of 66 °C. The D value could not be calculated for this isolate at 72 °C using linear regression as the curve tailed after the second time point leaving only the first two points from which a robust D value could not be calculated. This situation also occurred for *E. faecium* 2276 p1 at 78 °C. Analysis of Variance of the D values showed that there were no significant differences (p > 0.05) between the isolates or between the species at 72 °C.

3.3.2.4 z values
When the log_{10} D values were plotted against temperature, the fitted response line had good correlation coefficients (R) which were over 0.9 for all of the graphs. For the enterococci tested in this study, z values ranged from 5.0 to 9.8 °C (Table 3.3). The isolates with the lowest z values were all *E. faecalis*. Despite being less heat sensitive at the higher temperatures of 75 and 78 °C, *E. faecalis* 2356p1 from pasteurised milk had
similar z values to the *E. faecalis* Type strain. The isolates showing the greatest heat resistance belonged to the species *E. faecium, E. durans* and *E. hirae*.

### 3.3.2.5 Weibull model

Not all of the survival curves in the present study were linear, hence the Weibull model was applied to better interpret the data. Log\(_{10}\) reductions of the enterococci at 72 °C after 15 s were predicted using both the D value curves and the Weibull model (Table 3.4). They were generally predicted to be greater using the Weibull model, ranging from 0.10 to 3.33 log\(_{10}\) cfu/mL compared with 0.05 to 0.79 log\(_{10}\) cfu/mL using linear regression. When the model was used to predict the time required to obtain a 6 log\(_{10}\) cfu/mL reduction at 72 °C, the times ranged from 6 to 34 min. The isolates that reached a 6 log\(_{10}\) cfu/mL reduction the fastest did not necessarily have the greatest reductions after 15 s. For instance, *E. faecium* 2276p1 took the longest to reach a 6 log\(_{10}\) cfu/mL reduction but the reduction (Weibull) after 15 s was mid-range.

### 3.4 Discussion

When the thermoduric enterococci isolates were screened individually in BHIB for their ability to survive batch pasteurisation (thermotolerance ranking), the average log\(_{10}\) reduction of 61 *E. faecalis, E. faecium, E. durans* and *E. hirae* isolates was 1.1 log\(_{10}\) cfu/mL, with the most resistant isolates belonging to *E. durans*. In addition to heat resistance being influenced by species, it was also influenced by location. The majority of heat resistant *E. faecium* isolates were from one factory while the majority of heat sensitive isolates in general were from two other factories. Possible reasons for these variations may be due to different selective influences in the original environments, including exposure to different chemicals (including cleaning agents), heat and cold, all of which may have influenced thermal tolerance (Fernández et al., 2009). This highlights the importance of selecting isolates from multiple sources when determining variations in traits in populations and between species.
### Table 3.3 D and z values of pasteurised milk enterococci isolates

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>D values (min)</th>
<th>z values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>63 °C</td>
<td>69 °C</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>ATCC 19433</td>
<td>3.6 (0.97)</td>
<td>0.3 (0.97)</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>2350p1</td>
<td>49.0 (0.97)</td>
<td>NA</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>2356p1</td>
<td>18.4 (0.99)</td>
<td>1.3 (0.99)</td>
</tr>
<tr>
<td>E. faecium</td>
<td>2276p1</td>
<td>34.8 (0.99)</td>
<td>NT</td>
</tr>
<tr>
<td>E. faecium</td>
<td>2299p2</td>
<td>33.7 (0.99)</td>
<td>NT</td>
</tr>
<tr>
<td>E. durans</td>
<td>2128p1</td>
<td>29.0 (0.99)</td>
<td>NT</td>
</tr>
<tr>
<td>E. durans</td>
<td>2151p1</td>
<td>26.6 (0.99)</td>
<td>NT</td>
</tr>
<tr>
<td>E. hirae</td>
<td>2239p2</td>
<td>17.1 (0.97)</td>
<td>5.0 (0.98)</td>
</tr>
<tr>
<td>E. hirae</td>
<td>2452p2</td>
<td>19.1 (0.98)</td>
<td>NT</td>
</tr>
</tbody>
</table>

*a* Correlation coefficient (R) values in brackets, *b* NA = not applicable, *c* NT = not tested

### Table 3.4 Predicted log_{10} reduction of enterococci at 72 °C using Weibull and linear regression models

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>Log_{10} reduction (cfu/mL) in 15 s</th>
<th>Time (min) to obtain a 6 log_{10} reduction using the Weibull model</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. faecalis</td>
<td>2350p1</td>
<td>0.58</td>
<td>0.09</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>2356p1</td>
<td>2.25</td>
<td>0.79</td>
</tr>
<tr>
<td>E. faecium</td>
<td>2276p1</td>
<td>0.52</td>
<td>0.05</td>
</tr>
<tr>
<td>E. faecium</td>
<td>2299p2</td>
<td>0.54</td>
<td>0.16</td>
</tr>
<tr>
<td>E. durans</td>
<td>2128p1</td>
<td>0.20</td>
<td>0.09</td>
</tr>
<tr>
<td>E. durans</td>
<td>2151p1</td>
<td>0.10</td>
<td>0.08</td>
</tr>
<tr>
<td>E. hirae</td>
<td>2239p2</td>
<td>0.23</td>
<td>0.19</td>
</tr>
<tr>
<td>E. hirae</td>
<td>2452p2</td>
<td>0.30</td>
<td>0.05</td>
</tr>
</tbody>
</table>
The temperatures studied in the immersed coil procedure included 63 and 72 °C, as these temperatures are the minimum used in the batch (63 °C for 30 min) and HTST (72 °C for 15 s) pasteurisation of milk, respectively. Two further temperatures above 72 °C, at 3 °C intervals, were selected for inclusion in D value determinations. The higher temperature of 81 °C was not used as none of the isolates were detected after the first heating interval. This situation also occurred for *E. faecalis* 2356p1 and *E. hirae* 2239p2 at 78 °C, so the additional temperature of 69 °C was assessed for these two isolates.

Two isolates (*E. faecalis* 2356p1, *E. hirae* 2239p2) had lower D values at both the lowest (63 °C) and higher temperatures compared to the other isolates tested. *E. hirae* 2452p2 also had a lower D value at 63 °C compared to the other isolates but showed greater heat resistance in general as indicated by the higher $z$ value. In contrast, two other isolates (*E. faecalis* 2350p1, *E. faecium* 2276p1) exhibited greater heat resistance at 63 °C yet they were similar to the majority of isolates at the higher temperatures. This illustrates that heat resistance for particular isolates or species over a range of temperatures cannot necessarily be estimated from assessments at one or two temperatures.

Log_{10} reductions were calculated from the pasteurisation data (63 °C/30 min) of Rao et al. (1986) and Shannon et al. (1970) for *S. faecalis* and its subspecies *liquefaciens* and *zymogenes*. In contrast to the average log_{10} reduction of 1.3 log_{10} cfu/mL for *E. faecalis* (63 °C/30 min) in this study, the log_{10} reductions in the above mentioned studies were 2.5 and 4.2 log_{10} cfu/mL, respectively. The subspecies are now no longer recognized and are considered to be variations of *S. faecalis* (Schleifer, 1986), which has been renamed *Enterococcus faecalis* (Schleifer and Kilpper-Bälz, 1984). Additionally, Shannon et al. (1970) obtained a log_{10} reduction of 0.8 log_{10} cfu/mL for *S. durans* and indicated that it was the most heat resistant species from the isolates that they tested. This is comparable to the results from this study. Rao et al. (1986) obtained a log_{10} reduction of 0.2 log_{10} cfu/mL for *S. faecium*, which is considerably lower than the results presented here. However these researchers considered *S. durans* to be a subspecies of *S. faecium*, which would have affected any results attributable to species. *E. hirae* was first described in 1984 and isolates were possibly assigned to either *S. faecium* or *S. durans*.
previously (Devriese and Pot, 1995). The heat resistance of *E. hirae* has been infrequently characterised but one study found that it had a lower thermal resistance than *E. faecium* (Renner and Peters, 1999). In that study, *E. faecium* ATCC 6057 had a D value of 4.5 min at 65 °C whereas *E. hirae* ATCC 10541 had a D value of 1.5 min at 65 °C. Only one isolate of each species was assessed. The contrasting results for *E. hirae* in the current study highlight the importance of assessing multiple isolates from different sources for the characterisation of traits attributable to species.

Earlier studies on heat resistance employed methods that took from 20 s to five minutes for the test sample to reach the designated temperature (Shannon et al., 1970), which is not ideal when taking measurements at 15 s where any errors in the come-up time could be a significant portion of the test period. Some methods involved the preparation of multiple sample ampoules (Ghazala et al., 1995; Tait et al., 1991), which were manually removed from waterbaths. The preparation of multiple ampoules is time consuming while the manual removal of samples in several second intervals can introduce timing errors. The use of an immersed coil apparatus, such as the one employed in this study, overcomes these problems (Cole and Jones, 1990) by requiring the preparation of only one bulk sample, which is delivered at precise times and in precise volumes.

Comparison of the D values obtained in this study with those of other studies is difficult as the procedures, heating menstrua, and temperatures vary. The procedure used in the present study, using a submersed, heated coil will more closely resemble the situation in industry than previous laboratory methods. Also, several of the temperatures used in this study, 69, 72 and 78 °C, have not been reported in other work. The D values obtained for the Type strain *E. faecalis* ATCC 19433 in the current study are consistent with the log₁₀ reductions obtained for this strain in nutrient broth at 65 and 71 °C in the study by Bradley and Fraise (1996), and with D values obtained in Sorenson’s buffer (Magnus et al., 1986). In a trial using 3,3-Dimethylglutaric acid-sodium hydroxide buffer at pH 7 as the heated menstruum, *E. faecalis* had a D value of 1.3 min at 63 °C (Tait et al., 1991), which is considerably lower than the D values of 18.4 and 49.0 min of *E. faecalis* in BHIB in this study. BHIB has been recommended for use in thermal
resistance studies since it has a higher thermal resistance than buffer and it more closely resembles a food system (Simpson et al., 1994). On the other hand, the use of unbuffered BHIB as a growth medium could elicit an acid adaptation response that increases heat resistance, as has been shown with *E. faecium* (Fernández et al., 2009). In a study by Batish et al. (1988), BHIB was compared with full cream milk. At 63 °C, D values of *E. faecalis* were 7 to 17.5 min in full cream milk and 4.5 to 7.5 min in BHIB, while D values of *E. faecium* were 25.5 and 9 min in milk and broth, respectively. That study also found that increased milk fat influenced thermal resistance. Similarly, the heat resistance of *E. faecalis* was greater in whole milk than in a saline solution (Aguirre et al., 2009). This differs from other reports in which increased milk fat did not increase heat resistance (Kornacki and Marth, 1992; Shannon et al., 1970). Thermal resistance of *E. faecium* was increased in ground beef when the fat content was increased from 4 % to 12 %, but this effect only occurred at lower heat treatments (58-65 °C) and fat content was not a significant factor at the higher temperature of 68 °C (Ma et al., 2007). Milk was not used as the heating menstruum in the present study since the potential for fouling and blocking the small diameter heated coil during the long holding times at the lower treatment temperatures could have been problematic. However based on reported differences between BHIB and milk, some comparative testing to confirm or refute previous differences is warranted.

The other temperature common to this and other heat resistance studies was 75 °C. At this temperature, an isolate of *E. faecium* isolated from ham had a D value of 0.6 min in cooked meat broth (Ghazala et al., 1995) which was similar to the *E. faecium* isolate with median heat resistance in this study. *E. durans* ATCC 19432 had D values of 0.6 and 0.8 in BHIB (Simpson et al., 1994), although that study used selective media (KF *Streptococcus* agar and M-*Enterococcus* agar) to obtain post-heat treatment counts. Selective media may not be the best recovery media for bacteria that have undergone a stressful treatment, as some selective agents in the media may prevent recovery of injured cells. However, the use of selective media may be unavoidable in situations where there is a mixed bacterial population. The longer plate incubation time of 72 h required in the current study to obtain maximum survivor recovery suggests that
damage has occurred to the cells during the heating process that has necessitated additional recovery time compared with the typical 24 h incubation of healthy cells.

Comparisons of the $z$ values of *E. faecalis*, *E. faecium* and *E. durans* could be made with other published studies. In the present study, *E. durans* had $z$ values of 8.7 and 8.8 °C, which was slightly less than the $z$ values of 9.5-9.7 °C found in another study using BHIB as the heating menstruum (Simpson et al., 1994). Other researchers have obtained $z$ values of 5.2 °C for *E. faecalis* (Tait et al., 1991) and 4.5 °C for *E. faecium* (Martinez et al., 2003) in various buffers. When species obtained from cheese were compared for heat resistance in buffer, within one study, $z$ values were 2.5-3.2 °C for *E. faecalis*, 3.6 °C for *E. faecium* and 4.2 °C for *E. durans* (Sanz Perez et al., 1982). Although *E. durans* showed the greatest heat resistance, these values are all quite low compared with the results in this study. This may reflect the effects of growth and survival in different environments as the isolates in this study were obtained from laboratory pasteurised milk and therefore already possessed some heat resistance compared with other enterococci. Accordingly, enterococci isolated from pasteurised ham have been shown to have higher $z$ values, with *E. faecalis* and *E. faecium* having $z$ values of 7.2-8.1 °C and 8.1-8.3 °C in buffer, respectively (Magnus et al., 1986).

A factor, which was not investigated in this study, was the effect that initial growth temperature may have on the heat resistance of the cultures. For this study, cultures were initially grown at 37 °C, close to their optimum temperature. Martinez et al. (2003) found that an *E. faecium* isolate from cheese was more heat resistant when the culture was grown at lower temperatures (5 °C), particularly when the growth period was extended. Growth at 5 °C would more closely reflect the situation in the dairy industry, where milk is stored at approximately 4 °C or slightly higher if proper storage conditions have not been maintained. However, this other work only investigated thermal inactivation at 70 °C and the cultures were not chilled after heat treatment. The current Australian Food Standards Code for the processing of milk requires that pasteurisation be conducted for a minimum of 15 s at 72 °C, followed by immediate shock cooling to 4.5 °C (FSANZ, 2004). Cold shock can affect the recovery of enterococci (Hansen and Riemann, 1963) so it is an important step in the process. In the current study, cultures
were immediately cooled after heat treatment as this represents a condition of pasteurisation in the dairy industry. Further studies combining lower culture growth temperatures prior to pasteurisation, minimum 72 °C inactivation temperatures and post-treatment cooling would provide further information. Interestingly, Martinez et al. (2003) found that the phase of growth and the growth temperature of the cultures did not significantly (p > 0.05) affect $z$ values for *E. faecium*. If this follows true for other species, then the $z$ values obtained in the current study would not be different had the isolates been grown at 4 °C instead of 37 °C.

Both of the *E. hirae* curves at 63 °C exhibited shoulders, though this was not as noticeable for the other isolates, possibly because the other isolates, with the exception of *E. faecalis* 2356p1, did not have as great a total reduction by the last sampling time. Shoulders can be due to the tendency of the culture to form clumps, pairs or chains (Magnus et al., 1986). *E. hirae* 2452p2 did form cohesive, noticeable clumps in BHIB, which required longer vortexing in order to minimise counting inconsistencies on agar plates. At the lowest temperature studied here, the holding time was extensive, which would have enabled heat penetration of any clumps present. When the higher temperatures were assessed, the holding times were very short and heat penetration may not have extended through entire clumps. This could lead to a residual population inside the clump which had not received the full heat treatment and could then survive and appear to be more resistant to heat inactivation. This tendency to clump may have provided *E. hirae* 2452p2 with a certain degree of protection at the higher temperatures, allowing it to survive longer than the other more miscible cultures.

Although industry has traditionally utilised D values as a measure of the heat resistance of microorganisms, they can have limited value when microorganisms have non-linear survival curves. Linear regression works well when the survival curves decrease at a constant rate over time. In reality, microorganisms may exhibit shoulders at the beginning or tailing at the end of survival curves. Linear analysis does not fit well to such curves. This is indicated, in part, by lower correlation coefficient (R) values than for the more linear curves. At higher temperatures, culture numbers could have a rapid initial drop, followed by a gradual tailing. Other studies have found shoulders at the beginning.
of *E. faecium* (Ghazala et al., 1995; Magnus et al., 1988) and *E. faecalis* (Magnus et al., 1988) survival curves and variable tailing at the end of *E. faecium* survival curves (Ross et al., 1998). The tailing phenomena may be due to the presence of unequal heat resistance throughout the bacterial population (Hansen and Riemann, 1963). If D values are calculated from the slope generated by the initial rapid drop in microbial numbers, the D values would underestimate the number of survivors that may exist within a population. Likewise, if the D values are calculated from the slope following an initial shoulder, the number of survivors would be overestimated. Therefore it is important that both the shoulder and the tail end of curve are taken into consideration. The only accurate way to estimate heat resistance is with a fitted curve. For a more precise estimation of thermal death, the Weibull model has been used to successfully fit lines to non-linear survival curves (Mattick et al., 2001) and was used to fit curves to the present data. The model estimated that pasteurisation conditions produced only a 0.1 to 2.25 log$_{10}$ cfu/mL reduction and that the time required to obtain a 6 log$_{10}$ cfu/mL reduction at 72 °C was between 6 to 34 min for the more thermoduric enterococci found in dairy products. Since a pasteurisation time of several minutes is not feasible for industry, this indicates that a higher temperature is required if the more heat resistant enterococci are to be eliminated from raw milk. The presence of enterococci in milk post-pasteurisation could pose a food spoilage problem, as enterococci from ewes’ cheese and milk have been shown to have lipase activity (Semedo et al., 2003).
CHAPTER 4 - ABILITY OF ENTEROCOCCI TO SURVIVE AND GROW IN DAIRY PRODUCT ENVIRONMENTS
4.1 Introduction

Generally, enterococci are distinguished from other genera on their ability to grow under extreme conditions, including growth at 10 and 45 °C and in 6.5 % salt (Devriese and Pot, 1995). The potential to withstand a variety of stresses, such as exposure to temperature and pH extremes, and changes in osmolarity and water activity may allow enterococci to adapt to a variety of food environments (Morandi et al., 2005; Giraffa, 2014; Solheim et al., 2014). Milk and many dairy products are stored at temperatures under 10 °C and may also be subject to conditions with high acidity or salt concentrations. The response to environmental stresses in *E. faecalis* is influenced by the extracytoplasmic function sigma factor SigV, which is involved with responses to heat shock, exposure to ethanol and acidic conditions (Benachour et al., 2005). Heat shock proteins, activated by a sudden increase in temperature, can increase thermotolerance and salt tolerance (Ron, 2013) and have been identified as DnaK and GroEL in both *E. faecalis* and *E. faecium* (Boutibonnes et al., 1993; Laport et al., 2003). These proteins were involved in the heat shock response of *E. faecium*, which was able to survive at 70 °C for 2 h (Laport et al., 2003). This is close to but less than the pasteurisation temperature of 72 °C that was evaluated in Chapter 3.3.2.2. Nonetheless, the study of Laport et al. (2003) showed potential for the *E. faecium* Type strain ATCC 19434 to survive pasteurisation (72 °C, 15 s).

The presence of enterococci has been detected in a range of dairy products including butter, buttermilk, milk powder, yoghurt and ice cream from countries other than Australia, including India, South Africa and Pakistan using various methods of isolation and identification (Batish and Ranganathan, 1984a; Wessels et al., 1988; Javed et al., 2010). The earlier studies in 1984 and 1988 were conducted when the *Enterococcus* genus was newly described and only a few species were recognised (Schleifer and Kilpper-Bälz, 1984). Similarly, the study by Javed et al. (2010), which looked at butter, yoghurt and local processed cheese, only detected *E. faecalis* and *E. faecium*. Many of the studies of enterococci in food have focussed on cheese. Enterococci in cheese made from the milk of various animals including cows, goats, sheep and buffalo has been
discussed in a review by Foulquié Moreno et al. (2006). However, the majority of the studies on bovine milk as reported in that review, and in later work, investigated cheese made from raw milk. Throughout storage of European raw cow’s milk cheese, the predominant species isolated was *E. faecalis* followed by *E. faecium* in San Simón raw milk cheese after six weeks (García et al., 2002) and Montasio thermised (62 °C for 15 s) milk cheese (Marino et al., 2003). A study of semicotto caprino raw goat’s milk cheese found that *E. faecalis* was predominant in the cheese at the beginning of storage but that *E. faecium* predominated by 60 d storage (Suzzi et al., 2000). Enterococci are prevalent in raw milk, as found in the current work (Chapter 2) and in other studies (Garg and Mittal, 1991; Schlegelova et al., 2002; Kagkli et al., 2007). Provided that they survive the cheesemaking process, it is not surprising that they may be in cheese made from raw milk (Morandi et al., 2005; Moraes et al., 2012; Hammad et al., 2015). Cheddar cheese, which is made from pasteurised milk, is the main cheese produced in Australia, comprising approximately half of all cheese produced (Dairy Australia, 2015). Older studies observed that *E. durans* was more prevalent and survived better in Cheddar cheese than *E. faecalis*; however the cheesemilk was not pasteurised and had been heated to 63 °C without a holding period (Clark and Reinbold, 1966; Jensen et al., 1973). These studies were also conducted prior to the redesignation of faecal streptococci into the genus *Enterococcus* (Schleifer and Kilpper-Bälz, 1984), after which more species were attributed to this genus (Devriese and Pot, 1995; Giraffa, 2014). Few recent studies have looked at naturally occurring enterococci during storage of Cheddar cheese for more than a couple of months.

The prevalence and species of enterococci in pasteurised milk, butter, Cheddar cheese and milk powder shortly after manufacture is reported in Chapter 2. The detection of enterococci in these products was further assessed after typical storage periods. Pasteurised milk from one manufacturing plant had been tested over one year immediately after manufacture, to determine the survival rate of enterococci after factory pasteurisation (72 °C, 15 s) (Chapter 2.3.6.1). It was tested subsequently after two weeks storage of milk at 4 °C to ascertain whether very low numbers of survivors could grow over a typical shelf-life period, and in line with the generation times
measured for known thermoduric enterococci, to contribute to spoilage. In the present study, butter, Cheddar cheese and milk powder were stored at 4, 12 and 25 °C, respectively, for up to one year and assessed for changes in the levels of detection and species. In addition, the capacity of the thermoduric enterococci (Chapter 3.2.1) to grow at 4 and 7 °C, in 7-12 % salt and at pH levels of 3.5-6.0 in media, and in cottage cheese and yoghurt at 4 °C refrigeration temperatures, was also determined.

4.2 Materials and Methods

4.2.1 Assessment of enterococci growth in food environment conditions

4.2.1.1 Screening experiment at 4 and 7 °C
Sixty-one enterococci isolates from laboratory pasteurised milk (Chapter 3.2.1) were used in a preliminary investigation to assess ability to grow at 4 and 7 °C. The BHIB cultures (5 mL) were spotted onto duplicate BHIA plates using a 10 µL loop, placing 10 spots on each plate. Within 30 min of inoculation, plates were placed in a single layer into incubators to ensure equal temperature distribution. One replicate of each plate was incubated at 4 °C and the other at 7 °C for 10 d. Plates were then observed for the presence and extent of growth.

4.2.1.2 Growth of enterococci at different salt and pH levels
The thermoduric enterococci isolates (Chapter 3.2.2, Table 3.1) were assessed individually for their ability to grow in BHIB over a range of salt and pH levels. The BHI-based broths (10 mL) were prepared to contain the following: 0.5 (BHIB control), 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 % (w/v) NaCl, pH 7.4; or to have pH levels of 7.4 (BHIB control), 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0, adjusting with 4 M NaCl. Overnight BHIB cultures (5 mL) were diluted to a McFarland Standard 4 in 0.85 % saline and one loop (10 µL) used to inoculate the control and test broths. Cultures were prepared in quadruplicate and one set of each treatment was incubated at 4, 7, 10 and 37 °C. All cultures were assessed for visual turbidity at the following intervals: the 4 and 7 °C treatments were assessed for growth weekly for five weeks (35 d), the 10 °C treatment was assessed
biweekly for one month (28 d) and the 37 °C treatment was assessed daily for one week (7 d). Growth in the control broth (BHIB, 0.5 % NaCl, initial pH 7.4) indicated that growth at each temperature was possible. Test cultures were scored as showing when growth was visible or no growth at the end of the incubation period and the lowest pH or highest concentration of NaCl which allowed growth noted. Following the end of the incubation periods for each temperature, the broths were streaked onto BHIA to determine whether the salt and pH conditions that did not give rise to turbid growth were bacteriostatic or bacteriocidal.

4.2.2 Detection of enterococci during product storage

Dairy products were assessed for the presence of naturally occurring enterococci and were not inoculated with additional cultures. Pasteurised milk, butter, Cheddar cheese and milk powder samples were stored at temperatures and for periods typical for the products.

4.2.2.1 Pasteurised milk

Following testing on arrival for plate counts or enrichment culture, the remaining pasteurised milk samples (n = 22) containing approximately 1.9 L were stored at 4 °C for two weeks. The pasteurised milk was not inoculated with additional cultures and was assessed for the presence of naturally occurring enterococci at the end of shelf life (two weeks) at the legally required refrigeration temperature (≤ 5 °C). After storage, the samples were tested in CEB and on CEBA (Chapter 2.2.6.2). Results were recorded as the smallest volume of milk in which enterococci were detected (0.1 mL spread plate, 1, 10, 100 mL enrichment, or not detected in 100 mL), which provided a qualitative assessment of the minimum order of magnitude of cells present, and comparison made with the same sample when tested on arrival. This allowed an assessment of increases or decreases in viable enterococci present after storage in terms of change in $\log_{10}$ cfu/mL. A minimum of two presumptive enterococci isolates were taken from the streak plates of each positive sample, confirmed as enterococci (Chapter 2.2.6.5) and speciated (Chapter 2.2.6.6).
4.2.2.2 Butter, Cheddar cheese and milk powder

Butter was stored at 4 °C and tested at 0, 3 and 6 months while Cheddar cheese and milk powder were stored at 12 and 25 °C, respectively, and tested at 0, 6 and 12 months. Testing was conducted according to the procedure in Chapter 2 (Chapter 2.2.6.3). Results were recorded for the smallest sample size in which enterococci were detected (10 g, 100 g or not detected in 100 g). Increases or decreases in viable enterococci after storage were determined as described above and indicated a change in $\log_{10}$ cfu/g. Colonies were selected as indicated in Chapter 2.2.6.4.

4.2.3 Growth of enterococci in inoculated milk

The thermoduric enterococci isolates (Chapter 3.2.2, Table 3.1) were assessed for their ability to grow in milk at 4 and 7 °C to determine whether enterococci could grow in milk at typical transport and storage temperatures (4 °C or 40 °F) (Clemson University, 2015; Dairy Food Safety Victoria, 2015; Government of Canada, 2015) or at abuse temperatures likely to be found in domestic refrigerators (7 °C) (Evans and Redmond, 2015). The eight isolates were individually inoculated into 10 mL of BHIB from BHIA slopes and grown for 20 h at 37 °C. BHIB cultures were diluted in 0.1 % Bacteriological Peptone and both isolates of each species were inoculated into Ultra-High Temperature (UHT) whole milk (Devondale) to obtain a starting count of 30 to 90 cfu/mL. The inoculated milk was dispensed in 10 mL volumes into multiple MacCartney bottles for sampling at each time point and temperature. Milk incubated at 4 °C was tested weekly for five weeks. Milk incubated at 7 °C was tested biweekly for three weeks as the growth was expected to be quicker. Total plate counts were obtained using BHIA pour plates of 1 mL of milk and BHIA spiral plates (Chapter 3.2.2), in duplicate. Plates were incubated at 37 °C for 48 h and viable counts determined. Uninoculated UHT milk was incubated at each temperature and tested concurrently with the inoculated milk as a sterility control, using undiluted milk in pour plates.
4.2.4 Detection of enterococci in inoculated yoghurt and cheese

4.2.4.1 Yoghurt and cheese preparation
The thermoduric enterococci (Chapter 3.2.2, Table 3.1) were inoculated collectively into 10 g portions of plain, unflavoured yoghurt (10 % fat, pH 3.80, 77 mg salt/100 g) and 10 g portions of skim milk cottage cheese (4.5 % fat, pH 4.40, 28 mg salt/100 g) sourced from a local supermarket. The products were weighed into small (15 x 9.5 cm) stomacher bags (Seward, Worthing, West Sussex, UK). Each culture was diluted using 0.1 % Bacteriological peptone to approximately $10^5$ cfu/mL. The eight cultures were combined and 0.5 mL was added to each bag so that the final combined concentration of enterococci was approximately 50 000 cfu in 10 g of product (5000 cfu/g). This was greater than the concentration usually encountered in the products; however, this was necessary in order to be able to monitor enterococci counts throughout the storage period. Multiple bags were prepared to enable two bags of each product to be tested at each sampling period. Bags were incubated at 4 and 7 °C and sampled weekly for four weeks. Uninoculated samples of yoghurt and cheese were concurrently incubated as controls to enable comparisons between tests inoculated with enterococci versus the baseline microbiota in the products.

4.2.4.2 Yoghurt and cheese testing
Yoghurt and cottage cheese were prepared for testing following Australian Standards 1766.3.12 and 1766.3.15, respectively (Standards Australia 1992, 1994). At each sampling period, the product was diluted ten-fold. Yoghurt (10 g) was diluted with 90 mL 0.1 % Bacteriological Peptone and homogenised in a stomacher for one minute. Cottage cheese (10 g) was diluted with 50 mL 2 % sodium citrate (APS Finechem, Seven Hills, NSW, Australia) and 40 mL 0.1 % Bacteriological peptone. The cheese was homogenised in a stomacher for 90 s with the sodium citrate only and for a further 5 s after the addition of peptone. Appropriate dilutions were spread plated (0.1 mL), in duplicate, onto Slanetz and Bartley Medium (SBA; Oxoid). This agar was used since it inhibited the background microbiota in the yoghurt better than CEBA, while still allowing the selected test cultures to grow. SBA is the same formulation as MEA (see Chapter 2.2.3). Plates were incubated at 37 °C for 48 h and viable counts were determined.
(Standards Australia, 1991, AS 1766.1.4) on colonies exhibiting typical enterococci morphology (Chapter 2.3.1.1.2) (Figure 4.1). The uninoculated controls were tested in the same manner.

### 4.2.5 Analyses

Growth rates of enterococci in inoculated milk were determined using counts transformed to \( \log_{10} \) cfu/mL. The data was analysed using the DMFit models on the ComBase website (http://modelling.combase.cc/membership/ComBaseLogin.aspx) using the Baranyi and Roberts model (Baranyi and Roberts, 1994) to determine initial and final counts, and maximum growth rate.

Viable counts in the inoculated yoghurt and cheese were statistically analysed with Analysis of Variance using Genstat Release 9.1. Prior to analyses, the data was transformed to \( \log_{10} \).

### 4.3 Results

#### 4.3.1 Assessment of enterococci growth in food environment conditions

**4.3.1.1 Screening experiment at 4 and 7 °C**

All of the 61 enterococci isolated from laboratory pasteurised milk grew on BHIA plates at 4 and 7 °C, albeit some weaker than others (Table 4.1). Growth strength was subjectively assessed where strong growth was designated as dense culture growth on the plate and weak growth was a thin film of culture growth on the plate. Overall, 80 % of all the enterococci had strong growth on the BHIA plates at 4 °C, while 20 % showed weak growth. More specifically, only 5-11 % of *E. faecium*, *E. durans* and *E. hirae* isolates showed weak growth whereas 73 % of the 11 *E. faecalis* isolates showed weak growth. At 7 °C, 90 % of the enterococci isolates had strong growth while only 10 % had weak growth. The isolates with weaker growth at 7 °C were again predominantly *E. faecalis* (27 % of the isolates).
4.3.1.2 Growth of enterococci at different salt and pH levels

In these investigations, growth of enterococci in broth was determined as the time taken to reach visible turbidity under various conditions of incubation temperature, media pH and salt concentration.

4.3.1.2.1 Control media

In the control broths (BHIB, 0.5 % salt, pH 7.4), all eight of the thermoduric enterococci grew at 37 °C after one day and at 10 °C after four days, which were the first sampling times at each temperature (Tables 4.2, 4.3 and 4.4). At 7 °C, *E. faecalis* 2350p1 and *E. hirae* 2239p2 showed detectable growth after seven days, while the remaining cultures had detectable growth after 14 d (Table 4.5). Time taken to detect growth increased at 4 °C (Table 4.6). At this temperature, *E. faecalis* 2350p1, *E. faecium* 2299p2, *E. durans* 2151p1 and *E. hirae* 2239p2 and 2452p2 showed detectable growth after 14 d and *E. faecium* 2276p1 and *E. durans* 2128p1 after 21 d. *E. faecalis* 2356p1 did not show detectable growth after five weeks at 4 °C.

4.3.1.2.2 Salt

All eight isolates grew in 8.0 % salt at 37 °C after one to four days incubation. More than half the isolates, including the four *E. faecalis* and *E. hirae* isolates, and *E. faecium* 2276p1, grew in 8.0 % salt at 37 °C after one day (Table 4.2). *E. faecalis* 2356p1, *E. faecium* 2276p1 and *E. hirae* 2239p2 also grew in 9.0 % salt at 37 °C after a longer period of three to six days. None of the isolates grew in 10.0-12.0 % salt at 37 °C after incubation for seven days. Growth at different salt levels was temperature dependent and none of the isolates grew in 7.0-12.0 % salt at 4 and 7 °C after 35 d or at 10 °C after 28 d. Salt concentrations (7.0-12.0 %) were bacteriostatic for all of the isolates at 4-10 °C. At 37 °C however, 12 % salt was bacteriocidal for *E. hirae* 2239p2 and 9.0-12.0 % salt was bacteriocidal for *E. hirae* 2452p2. All of the salt concentrations were bacteriostatic for the *E. faecalis, E. faecium* and *E. durans* isolates.
Figure 4.1 *E. faecalis* on SBA

Table 4.1 Percentage of pasteurised milk isolates with weak or strong growth at 4 and 7 °C

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of isolates</th>
<th>Weak growth (%)</th>
<th>Strong growth (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4 °C 7 °C</td>
<td>4 °C 7 °C</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>11</td>
<td>73 27</td>
<td>27 73</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>22</td>
<td>9 5</td>
<td>91 96</td>
</tr>
<tr>
<td><em>E. durans</em></td>
<td>19</td>
<td>5 5</td>
<td>95 95</td>
</tr>
<tr>
<td><em>E. hirae</em></td>
<td>9</td>
<td>11 11</td>
<td>89 89</td>
</tr>
</tbody>
</table>

Table 4.2 Detection of enterococci growth in BHIB with 7.0-12.0 % salt at 37 °C

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>Time to detection of growth at each salt level (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C¹ 7.0 % 8.0 % 9.0 % 10.0 % 11.0 % 12.0 %</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>2356p1</td>
<td>1 1 1 4 ND² ND ND</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>2350p1</td>
<td>1 1 1 ND ND ND ND</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>2299p2</td>
<td>1 2 3 ND ND ND ND</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>2276p1</td>
<td>1 1 1 3 ND ND ND ND</td>
</tr>
<tr>
<td><em>E. durans</em></td>
<td>2151p1</td>
<td>1 1 1 3 ND ND ND ND</td>
</tr>
<tr>
<td><em>E. durans</em></td>
<td>2128p1</td>
<td>1 1 1 4 ND ND ND ND</td>
</tr>
<tr>
<td><em>E. hirae</em></td>
<td>2452p2</td>
<td>1 1 1 ND ND ND ND</td>
</tr>
<tr>
<td><em>E. hirae</em></td>
<td>2239p2</td>
<td>1 1 1 6 ND ND ND</td>
</tr>
</tbody>
</table>

¹C = control broth, BHIB 0.5 % NaCl, pH 7.4

²ND = growth not detected after 7 d
Table 4.3 Detection of enterococci growth in BHIB at pH 3.5-6.0 at 37 °C

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>Time to detection of growth at each pH level (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C¹ 6.0 5.5 5.0 4.5 4.0 3.5</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>2356p1</td>
<td>1 1 1 1 1 1 ND² ND ND</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>2350p1</td>
<td>1 1 1 1 1 1 ND ND ND</td>
</tr>
<tr>
<td>E. faecium</td>
<td>2299p2</td>
<td>1 1 1 1 1 ND ND ND</td>
</tr>
<tr>
<td>E. faecium</td>
<td>2276p1</td>
<td>1 1 1 1 ND ND ND ND</td>
</tr>
<tr>
<td>E. durans</td>
<td>2151p1</td>
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<td>E. durans</td>
<td>2128p1</td>
<td>1 1 1 1 3 ND ND ND ND</td>
</tr>
<tr>
<td>E. hirae</td>
<td>2452p2</td>
<td>1 1 1 1 ND ND ND ND</td>
</tr>
<tr>
<td>E. hirae</td>
<td>2239p2</td>
<td>1 1 1 1 1 ND ND ND ND</td>
</tr>
</tbody>
</table>

¹C = control broth, BHIB 0.5 % NaCl, pH 7.4
²ND = growth not detected after 7 d

Table 4.4 Detection of enterococci growth in BHIB at pH 3.5-6.0 at 10 °C

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>Time to detection of growth at each pH level (d)</th>
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<tbody>
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<td></td>
<td></td>
<td>C¹ 6.0 5.5 5.0 4.5 4.0 3.5</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>2356p1</td>
<td>4 4 4 7 ND ND ND ND</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>2350p1</td>
<td>4 4 4 7 ND ND ND ND</td>
</tr>
<tr>
<td>E. faecium</td>
<td>2299p2</td>
<td>4 4 4 7 ND ND ND ND</td>
</tr>
<tr>
<td>E. faecium</td>
<td>2276p1</td>
<td>4 4 7 14 ND ND ND ND</td>
</tr>
<tr>
<td>E. durans</td>
<td>2151p1</td>
<td>4 4 4 7 ND ND ND ND</td>
</tr>
<tr>
<td>E. durans</td>
<td>2128p1</td>
<td>4 4 7 ND ND ND ND ND</td>
</tr>
<tr>
<td>E. hirae</td>
<td>2452p2</td>
<td>4 4 4 7 ND ND ND ND</td>
</tr>
<tr>
<td>E. hirae</td>
<td>2239p2</td>
<td>4 4 4 4 18 ND ND ND ND</td>
</tr>
</tbody>
</table>

¹C = control broth, BHIB 0.5 % NaCl, pH 7.4
²ND = growth not detected after 28 d.

Table 4.5 Detection of enterococci growth in BHIB at pH 3.5-6.0 at 7 °C

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>Time to detection of growth at each pH level (d)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>C¹ 6.0 5.5 5.0 4.5 4.0 3.5</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>2356p1</td>
<td>14 14 14 28 ND² ND ND ND</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>2350p1</td>
<td>7 7 14 28 ND ND ND ND</td>
</tr>
<tr>
<td>E. faecium</td>
<td>2299p2</td>
<td>14 14 14 14 14 ND ND ND</td>
</tr>
<tr>
<td>E. faecium</td>
<td>2276p1</td>
<td>14 21 21 28 ND ND ND ND</td>
</tr>
<tr>
<td>E. durans</td>
<td>2151p1</td>
<td>14 14 14 21 ND ND ND ND</td>
</tr>
<tr>
<td>E. durans</td>
<td>2128p1</td>
<td>14 14 14 14 ND ND ND ND</td>
</tr>
<tr>
<td>E. hirae</td>
<td>2452p2</td>
<td>14 14 14 14 ND ND ND ND</td>
</tr>
<tr>
<td>E. hirae</td>
<td>2239p2</td>
<td>7 7 7 14 ND ND ND ND</td>
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</tbody>
</table>

¹C = control broth, BHIB 0.5 % NaCl, pH 7.4
²ND = growth not detected after 35 d.
4.3.1.2 pH

At 37 °C, the majority of the enterococci grew in media of pH 4.5 within three days (Table 4.3), with the lowest pH that allowed growth occurring between 4.0 and 4.5 (Figure 4.2). At 10 and 7 °C, the pH level that allowed growth was usually higher, between 4.5 and 5.0. Growth at pH 5.0 was achieved after seven days at 10 °C, but took 14-28 d at 7 °C, depending on the isolate (Tables 4.4 and 4.5). The lowest pH level that allowed growth at 4 °C ranged from pH 5.0 to 5.5 for six of the isolates (Figure 4.2). The time taken to detect the growth of *E. faecalis*, *E. durans* and *E. faecium* 2299p2 isolates at pH 5.5 was 14-21 d (Table 4.6). Both *E. hirae* isolates grew at pH 5.0 after 21-28 d incubation. *E. faecium* 2276p1 was most sensitive to pH at 4 °C. It grew in the BHIB control (pH 7.4) but not at pH 6.0 at 4 °C after 35 d even though growth was detected at pH 5.0 at 7 °C after 28 d.

At every temperature, pH 3.5 was bacteriocidal for all of the isolates except *E. faecium* 2276p1, where pH 3.5 was bacteriostatic at 4, 7 and 10 °C. *E. faecium* 2276p1 was most sensitive to pH at the lowest temperature (4 °C) (Figure 4.2). The BHIB was bacteriocidal at pH 4.0 for all of the isolates at 37 °C but not at the other temperatures, indicating that lower temperatures offered the enterococci protection from the lethal effects of acidity. Further bacteriocidal effects at 37 °C were seen with *E. faecalis* 2356p1, *E. faecium* 2276p1 and *E. hirae* 2452p2 at pH 4.5 and *E. faecalis* 2356p1 at pH 5.0.

4.3.2 Detection of enterococci during product storage

4.3.2.1 Pasteurised milk

Twenty-two of the 24 of pasteurised milk samples were stored for two weeks at 4 °C and then tested using plate count and enrichment procedures to detect enterococci potentially present initially at levels of <1 cfu/100 mL (Table 4.7). Calculations, based on the lowest growth rate of the thermoduric enterococci isolated in this study (Table 4.8), indicate the two week storage period provided sufficient generation times to enable detection of enterococci at a level of at least 1 cfu/100 mL (and even as low as 1 cfu/L).
Table 4.6 Detection of enterococci growth in BHIB at pH 3.5-6.0 at 4 °C

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>Time to detection of growth at each pH level (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C¹</td>
<td>6.0</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>2356p1</td>
<td>14</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>2350p1</td>
<td>14</td>
</tr>
<tr>
<td>E. faecium</td>
<td>2299p2</td>
<td>14</td>
</tr>
<tr>
<td>E. faecium</td>
<td>2276p1</td>
<td>21</td>
</tr>
<tr>
<td>E. durans</td>
<td>2151p1</td>
<td>14</td>
</tr>
<tr>
<td>E. durans</td>
<td>2128p1</td>
<td>21</td>
</tr>
<tr>
<td>E. hirae</td>
<td>2452p2</td>
<td>14</td>
</tr>
<tr>
<td>E. hirae</td>
<td>2239p2</td>
<td>14</td>
</tr>
</tbody>
</table>

¹C = control broth, BHIB 0.5 % NaCl, pH 7.4
²ND = growth not detected after 35 d.

Figure 4.2 Lowest pH level at which growth of enterococci was detected at 4, 7, 10 and 37 °C

Growth was assessed in 0.5 pH increments. The figure shows the lowest pH level at which growth was detected at each temperature.

*At 4°C, the lowest pH that allowed growth of 2276p1 was between 6.0 and 7.4
Three of the 22 pasteurised milk samples had detectable enterococci initially. Enterococci were detected in 40.9 % of pasteurised milk samples after two weeks storage (Table 4.7). The majority of these detections occurred in 100 mL enrichments (22.7 %); however, enterococci were detected in four enrichments of less than 100 mL. One sample had a measurable count of 2.84 log$_{10}$ cfu/mL on a CEBA spread plate, where the prevalent species, *E. durans*, was the main species found in milk immediately after pasteurisation (Table 4.7). This level of viable count is consistent with the measured generation time for this species (Figure 4.3, Table 4.8), the starting count of 0.01 to 0.09 log$_{10}$ cfu/mL and incubation period of two weeks at 4 °C. Enterococci were not detected in 54.5 % of the samples either following manufacture or after two weeks storage, as they were below the limit of detection (< 1 cfu in 100 mL).

Immediately after factory pasteurisation, *E. durans* and *E. malodoratus* were detected at 0.01 to 0.09 cfu/mL, whereas after storage at 4 °C for two weeks, four other species in addition to *E. durans* (*E. faecalis*, *E. hirae*, *E. asini* and *E. sulfureus*) were detected in nine samples and *E. malodoratus* was not detected (Table 4.7). This indicated that very low numbers of several thermoduric enterococci had survived pasteurisation and were able to grow in milk at 4 °C to detectable numbers. The 10 and 1 mL enrichments contained *E. asini* and the 0.1 mL spread plate contained *E. durans*. *E. faecium* was not detected in the pasteurised milk initially or after storage.

4.3.2.2 Butter, Cheddar cheese and milk powder

Butter, Cheddar cheese and milk powder samples were monitored for the presence or absence of enterococci in 10 and 100 g over typical commercial storage periods. The proportion of samples showing a one log$_{10}$ cfu/g increase or decrease or no change in detection level after storage relative to the levels detected after manufacture is shown in Table 4.9. In butter after three months, enterococci did not increase in the majority of the butter samples, although almost a third of the samples had a one log$_{10}$ cfu/g decrease. By six months, the majority of samples (68.8 %) had at least a one log$_{10}$ cfu/g decrease in enterococci compared to the levels detected after manufacture. At this time, nine of the 10 salted butter samples had a decrease in enterococci levels.
Table 4.7 Detection of enterococci and incidence of species type following factory pasteurisation of milk and following two weeks incubation of milk samples at 4 °C

<table>
<thead>
<tr>
<th>Time after pasteurisation (days)</th>
<th>Number of samples(^1) positive in the range (min cfu/mL detectable)(^2)</th>
<th>Incidence of species in samples (% of positive samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ND(^3) 0.01 0.1 1 10</td>
<td></td>
</tr>
</tbody>
</table>
| 0                                | 19 5\(^4\) 0 0 0                              | E. durans (80 %)
E. malodoratus (20 %)                                                   |
| 14                               | 13 5 2 1 1\(^5\)                             | E. sulfureus (44.4 %)
E. asini (33.3 %)\(^6\)
E. durans (33.3 %)\(^7\)
E. hirae (22.2 %)
E. faecalis (11.1 %)                                                   |

\(^1\) 24 samples of pasteurised bulk milk were collected across season from Factory B for plate counts; 22 of these samples were enriched to detect enterococci immediately after collection then incubated for two week at 4 °C before retesting by plate counts and enrichment.

\(^2\) Minimum number of cells/mL detectable by enriching of 100 mL, 10 mL, or 1 mL of pasteurised milk in CEB broth or by direct plating of 0.1 ml on spread plates of CEBA

\(^3\) ND = none detected (< 1 cell/100 mL of milk)

\(^4\) 3 E. durans in autumn samples, 1 E. durans in summer and 1 E. malodoratus in winter

\(^5\) CEBA spread plate count average 2.82 log\(_{10}\) cfu/mL

\(^6\) E. asini detected in 1 and 10 mL enrichments

\(^7\) E. durans detected in 0.1 mL spread plates
to levels immediately after manufacture, whereas only two of the six unsalted butter samples (33%) had a decrease in enterococci levels. In the 15 butter samples containing enterococci, the majority of the species detected at every testing period were *E. faecalis*. *E. casseliflavus* and *E. hirae* were only detected after manufacture and after three months, respectively, while *E. faecium* was detected throughout the storage period. At the end of storage, the number of samples in which *E. faecalis* was detected decreased from 12 to five, whereas *E. faecium* was detected in three samples both after manufacture and after six months.

Enterococci were detected in seven of the eight Cheddar cheeses. The level of enterococci either decreased or had no change in one log$_{10}$ cfu/g after both six and 12 months compared to after manufacture (Table 4.9). At six months, 75% of the samples had no change and 25% had a decrease by at least one log$_{10}$ cfu/g, but at 12 months, only 37.5% of the samples had no change while 62.5% of the samples had a decrease by at least one log$_{10}$ cfu/g. Enterococci did not have a one log$_{10}$ cfu/g increase after either six or 12 months. The predominant species isolated from the Cheddar cheese was *E. malodoratus*, which was the only species detected after 12 months. The most diverse species range occurred at the start of storage. By six months, *E. faecalis* and *E. faecium* were no longer detected in the Cheddar cheese.

Enterococci were detected in 12 of the 14 milk powder samples. After manufacture, four samples contained enterococci in 100 g and six samples contained enterococci in 10 g of milk powder. The remaining four samples did not contain enterococci in 100 g of milk powder after manufacture. Enterococci were not detected in two of these four samples after either six or 12 months (Table 4.9). In the other two samples, enterococci were detected in 100 g of one sample after six months only and in 100 g of the other sample after 12 months. At six months, enterococci levels remained unchanged in more than half of the samples (64.3%). After 12 months, over one third of the samples had at least a one log$_{10}$ cfu/g decrease in enterococci. *E. faecium* was the sole species detected in the milk powder samples throughout the storage trial.
Table 4.8 Parameters of *Enterococcus* spp. growth curves at 4 and 7 °C in milk determined from DMFit models

<table>
<thead>
<tr>
<th>Enterococcus species</th>
<th>Incubation Temperature (°C)</th>
<th>Initial Value(^1) (log(_{10}) cfu/mL)</th>
<th>Final Value (log(_{10}) cfu/mL)</th>
<th>Maximum Rate(^2) (log(_{10}) cfu/mL per hour) X 100</th>
<th>Generation Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. faecalis</td>
<td>4</td>
<td>1.55</td>
<td>5.92</td>
<td>0.75</td>
<td>39.8</td>
</tr>
<tr>
<td>E. faecium</td>
<td>1.68</td>
<td>6.42</td>
<td>0.73</td>
<td>41.0</td>
<td></td>
</tr>
<tr>
<td>E. hirae</td>
<td>1.48</td>
<td>7.10</td>
<td>0.79</td>
<td>37.6</td>
<td></td>
</tr>
<tr>
<td>E. durans</td>
<td>1.47</td>
<td>6.76</td>
<td>0.85</td>
<td>35.5</td>
<td></td>
</tr>
<tr>
<td>E. faecalis</td>
<td>7</td>
<td>1.75</td>
<td>7.41</td>
<td>1.91</td>
<td>15.9</td>
</tr>
<tr>
<td>E. faecium</td>
<td>1.82</td>
<td>8.10</td>
<td>1.48</td>
<td>20.8</td>
<td></td>
</tr>
<tr>
<td>E. hirae</td>
<td>1.96</td>
<td>7.67</td>
<td>1.37</td>
<td>22.0</td>
<td></td>
</tr>
<tr>
<td>E. durans</td>
<td>1.51</td>
<td>8.01</td>
<td>1.65</td>
<td>19.5</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Inoculated level

\(^2\) All curves had R values above 0.99
Table 4.9 Detection of enterococci in milk products after storage at typical storage temperatures

<table>
<thead>
<tr>
<th>Product</th>
<th>Three months</th>
<th></th>
<th></th>
<th>Six months</th>
<th></th>
<th></th>
<th>Twelve months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IN&lt;sup&gt;1&lt;/sup&gt;</td>
<td>NC&lt;sup&gt;2&lt;/sup&gt;</td>
<td>DC&lt;sup&gt;3&lt;/sup&gt;</td>
<td>IN</td>
<td>NC</td>
<td>DC</td>
<td>IN</td>
</tr>
<tr>
<td>Butter n = 16</td>
<td>12.5</td>
<td>56.3</td>
<td>31.3</td>
<td>6.3</td>
<td>25.0</td>
<td>68.8</td>
<td></td>
</tr>
<tr>
<td>Cheddar cheese n = 8</td>
<td>0.0</td>
<td>75.0</td>
<td>25.0</td>
<td>0.0</td>
<td>37.5</td>
<td>62.5</td>
<td></td>
</tr>
<tr>
<td>Milk powder n = 14</td>
<td>7.1</td>
<td>64.3</td>
<td>28.6</td>
<td>7.1</td>
<td>57.1</td>
<td>35.7</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Increase (%) in detection level of enterococci in samples after storage compared to after manufacture

<sup>2</sup> No change (%) in detection level of enterococci in samples after storage compared to after manufacture

<sup>3</sup> Decrease (%) in detection level of enterococci in samples after storage compared to after manufacture


4.3.3 Growth of enterococci in inoculated milk

Figure 4.3 shows the growth rates of *E. faecalis*, *E. faecium*, *E. hirae* and *E. durans* isolates when cultured in UHT milk at 4 and 7 °C with inocula containing two strains of the same species. The viable count numbers represent the total for both strains in the one culture. The lag phase was longer for all isolates at 4 °C, with viable counts of *E. faecalis*, *E. faecium* and *E. durans* remaining unchanged after the first week at 4 °C and only increased by 0.5 to 1.0 log$_{10}$ cfu/mL after two weeks. Since the stationary phase did not appear to be reached for *E. faecalis*, *E. faecium* and *E. durans* at 4 °C, there was potential for further growth of these isolates if the incubation period had continued. At 7 °C, all cultures achieved a maximum viable count between 7.4 to 8.1 log$_{10}$ cfu/mL within three weeks. The generation times for the species tested were 35.5 to 41.0 h at 4 °C and 15.9 to 22.0 h at 7 °C (Table 4.8).

4.3.4 Detection of enterococci in inoculated yoghurt and cheese

The pH of the products was 4.1 for the yoghurt and 4.4 for the cottage cheese, while the product specifications listed the salt content at 0.08 % and 0.03 %, respectively. The mixture of enterococci (two isolates each of *E. faecalis*, *E. faecium*, *E. hirae* and *E. durans*) inoculated into yoghurt and cottage cheese was able to survive but not grow in both products at 4 and 7 °C after storage for four weeks (Figure 4.4). Counts of enterococci in the cottage cheese samples remained unchanged and were not significantly different between either of the two storage temperatures or of the five sampling periods (p > 0.05). In the yoghurt samples, enterococci numbers decreased significantly (p = 0.005) during storage at both temperatures. At 4 °C, the counts dropped significantly (p = 0.005) after three weeks whereas the counts were significantly lower (p = 0.005) after two weeks at 7 °C.
Figure 4.3 Growth of *Enterococcus* species at 4 and 7 °C in inoculated milk

- ○ *E. faecalis* 4 °C
- ● *E. faecalis* 7 °C
- □ *E. faecium* 4 °C
- ■ *E. faecium* 7 °C
- △ *E. hirae* 4 °C
- ▲ *E. hirae* 7 °C
- ◇ *E. durans* 4 °C
- ♦ *E. durans* 7 °C

Shaded areas - product not tested at these storage times

Figure 4.4 Survival of *Enterococcus* species at 4 and 7 °C in cheese and yoghurt

- ☐ Cheese 4 °C
- ■ Cheese 7 °C
- □ Yoghurt 4 °C
- □ Yoghurt 7 °C

LSD= Least Significant Difference, p < 0.05

98
4.4 Discussion

Limited consortia of 4 species that had previously been shown to be heat resistant (Chapter 3.3.1) showed that these species could grow at 4 °C and at elevated refrigeration temperatures (7 °C) likely to be encountered in domestic fridges (Evans and Redmond, 2015). Growth at 10 °C is one of the defining characteristics of the original description of the Enterococcus genus (Devriese and Pot, 1995) but growth at 4 to 7 °C refrigeration temperatures has not been characterized previously. Growth kinetics showed a lag period for some cultures and some variation in growth rate, noting the growth rate was significantly slower at 4 °C relative to 7 °C (Chapter 4.3.2.1, Table 4.8). Knowing the approximate doubling time of the species at 4 °C was useful in understanding the incubation time that would be needed to detect very small numbers of survivors of pasteurisation – 0.01-0.09 cells/mL. Understanding the growth kinetics of thermoduric enterococci for the species not investigated here may inform setting shelf-life periods to minimise spoilage. Further work could be undertaken to understand the growth kinetics of other thermoduric enterococci.

Differences in the range of enterococci species detected in the dairy products were apparent. E. faecalis and E. faecium were the main species detected throughout butter storage, but at the end of storage, the prevalence of E. faecalis had decreased while the prevalence of E. faecium remained relatively unchanged (Chapter 4.3.2.2). This suggests that E. faecium has a better tolerance to the oil and water emulsion environment of butter than E. faecalis. While butter contains 1.5-2 % salt overall, the salt concentration is between 11-12.5 % in the aqueous phase of salted butter, which is where the bacteria are situated, although unsalted butter is more unstable microbiologically (ICMSF, 2005). The growth and spread of bacteria in butter is influenced by the water droplet size throughout the butter; growth of bacteria will be limited and die-off may occur following storage if the water droplets are small and well dispersed, but pH and salt in the aqueous phase become important preservation factors if butter contains coarse water droplets and water channels.
(ICMSF, 2005). In the current study, the presence of salt may have influenced the decrease in enterococci levels during storage of butter samples after six months, although a salt concentration of 11-12 % was bacteriostatic for the enterococci studied at refrigerated temperatures in BHIB (Chapter 4.3.1.2.2). The increase in enterococci levels in a limited number of butter samples examined indicated that there was potential for spoilage of butter by enterococci.

The presence and species of enterococci in milk powder after storage has not been previously reported. An earlier study from India which looked at the presence of enterococci in bovine non-fat dry milk on one occasion found both *E. faecalis* and *E. faecium*, although *E. faecalis* was more prevalent (Batish and Ranganathan, 1984b). On average, *E. faecium* comprised 8.6 % of the enterococci isolated in the raw milk (Chapter 2.3.5) yet it was the sole species detected in the milk powder throughout storage in the current study (Chapter 4.3.2.2), which may indicate that this species not only displays heat resistance but also has some resilience to desiccation. Milk powder is dry and only has a water activity of 0.3-0.4 (ICMSF, 2005b). Microbial growth will usually not occur in dry milk powder; therefore it was not unexpected that the level of enterococci did not generally increase during the current storage trial. However, both *E. faecium* and *E. faecalis* have been found to grow in the last part of the evaporator section or in the balance tanks of milk powder plants and to survive the spray drying process (Stadhouders et al., 1982). Likewise, thermoduric *E. durans* has been isolated from whey protein concentrate (Walsh et al., 2012) and *E. hirae* and *E. faecalis* have survived the spray drying process in a skim milk suspension (Ilango et al., 2016). This shows that if thermoduric enterococci are in the milk prior to spray drying, they have the ability to survive the process into the product.

In the Cheddar cheese, the detection level of enterococci was unchanged in the majority of samples at six months (Chapter 4.3.2.2, Table 4.9). Similarly, an Irish study reported enterococci counts remained relatively constant in a Cheddar-type cheese after 8 weeks (Gelsomino et al., 2001), although results were not presented past this time. Other studies using enterococci as starter adjuncts in Cheddar cheese
found that enterococci counts remained relatively constant for even longer periods of 9-12 months (Gardiner et al., 1999; Foulquié Moreno et al., 2003; Rea et al., 2004). This is in contrast to the enterococci in the Cheddar cheese in the current work, where there was a decrease in the detection level of enterococci in the majority of samples after 12 months (Chapter 4.3.2.2). This could be attributable to the particular strains of enterococci in the cheese as well as the influence of other lactic acid bacteria (LAB). Non-starter LAB (NSLAB) are the predominant microorganisms in cheese as they can cope with the nutritional stresses in the cheese environment, and increase in number during Cheddar cheese ripening (Gardiner et al., 1999; Rea et al., 2004; Settanni and Moschetti, 2010). When three Cheddar-type cheese manufactures were evaluated, the trial in which NSLAB reached the highest numbers coincided with lower levels of enterococci in the cheese (Gelsomino et al., 2001), suggesting that the enterococci could be out-competed by faster growing LAB. Other microbiota, including LAB, were not measured in the current work but could be a reason why the enterococci decreased in level of detection.

_E. malodoratus_ was the predominant species in the Cheddar cheese and was the only species detected at the end of storage (Chapter 4.3.2.2), although it occurred infrequently in raw milk (Chapter 2.3.5) and was not detected in the pasteurised milk (Chapter 4.3.2.1, Table 4.7). This suggests that this species may be particularly adapted to the cheese environment and perhaps this is not surprising since _E. malodoratus_ was originally isolated from Gouda cheese (Devriese and Pot, 1995). _E. malodoratus_ has infrequently been reported in food but it was one of the main species found in a study of Spanish soft and hard goat milk cheeses (Martín-Platero et al., 2009) and has been isolated from various Greek goats’ and ewes’ milk cheeses, San Simón raw cow’s milk cheese and Robiola di Roccaverano cheese made from a combination of pasteurised cow and raw goats’ milk (García et al., 2002; Bonetta et al., 2008; Litopoulou-Tzanetaki and Tzanetakis, 2011, 2014). In other cheese studies, enterococci found in an Irish raw cows’ milk Cheddar-type cheese were _E. casseliflavus_ (75 %) and _E. faecalis_ (25 %) (Gelsomino et al., 2001). However, _E. casseliflavus_ was the predominant species found in the raw milk of the Irish study,
while it only comprised 3.5 % of the raw milk enterococci in this study (Chapter 2.3.5). Furthermore, the Cheddar cheese in this trial was made from bulk pasteurised milk, whereas the Irish study looked at cheese made from raw milk solely from one farm. Other studies of enterococci in cheese have focused mainly on various European cheese varieties often made with milk from animals other than cows. Pesavento et al. (2014) found that the enterococcal species isolated from cow’s milk mozzarella cheese were 62.3 % *E. faecium*, 32.4 % *E. durans* and only 5.4 % *E. faecalis*. Interestingly, a French study compared enterococci in cow, sheep and goat milk cheese made from both raw milk and pasteurised milk. While enterococci were detected in 23 % of the pasteurised milk cheeses, they were isolated from 68 % of the raw milk cheeses, with the majority of the enterococci being *E. faecalis* (Jamet et al., 2012). Further to this, the cheese containing the higher levels of enterococci (10⁴-10⁶ cfu/g) were found in significantly more raw milk cheeses (53 %) compared to pasteurised milk cheeses (17 %). A similar finding was made in Belgian Herve cheese in which enterococci were found in greater numbers in the raw milk cheese but were barely detected in pasteurised milk cheese (Delcenserie et al., 2014). This adds another layer of complexity to the presence of enterococci in food, as many of the artisanal European cheeses are made from raw milk. Without a pasteurisation or heat inactivation step, enterococci that are present in raw milk are more likely to occur in the final product. The heat resistance of *E. malodoratus* is unknown and may be a reason why it predominates in the Australian Cheddar cheese. Limited information is available on the presence of *E. malodoratus* in relation to NSLAB during cheese ripening. Further knowledge in this area may explain why there was a decreased level of detection of enterococci in the cheese at the end of storage.

Enterococci grew in higher salt concentrations and lower pH levels at 37 °C than they did at the lower temperatures (4, 7 and 10 °C) (Chapter 4.3.1.2.2 and Chapter 4.3.1.2.3). However the higher incubation temperature was also more bacteriocidal at acidic and higher salt conditions than the lower temperatures. The enterococci were incubated for 35 d at 4 and 7 °C, but it is unknown whether or not a longer incubation period would have influenced the maximum salt and lowest pH levels in
which enterococci growth could be detected. The pH appeared to play a more important role than salt concentration in limiting the growth of enterococci in refrigerated conditions since highly acidic conditions were bacteriocidal to the enterococci at every temperature, whilst high salt concentrations were bacteriostatic at cold storage temperatures (4, 7 and 10 °C). In a study on the effects of pH, salt and incubation temperature on an isolate of *E. faecalis*, the most important factor influencing the growth of the culture was found to be pH (Gardini et al., 2001). In the present study, this was illustrated by the addition of enterococci to yoghurt and cottage cheese, which were stored at 4 and 7 °C for one month. Whilst the salt content of the products was negligible, the yoghurt and cheese had pH levels of 4.1 and 4.4, respectively. The enterococci were able to survive but not grow in the products at these conditions and began to decrease in number after storage in the more acidic yoghurt where competing yoghurt microbiota may also have had an impact.

### 4.5 Conclusion

Thermoduric enterococci were able to grow at 4 and 7 °C in UHT milk, although *E. faecalis* showed weaker growth than *E. faecium*, *E. hirae* and *E. durans* on agar at both of these temperatures. When milk inoculated with $10^2$ cfu/mL was stored at 7 °C, enterococci numbers reached spoilage levels in two weeks. This demonstrates the potential ability of enterococci which can survive pasteurisation to be spoilage bacteria if milk is stored above the recommended refrigeration temperature of 4 °C. The ability of the enterococci to grow in acidic conditions decreased as the incubation temperatures decreased from 37 °C down to 4 °C. Conversely, acidic conditions were bacteriostatic at 4 °C but bacteriocidal at 37 °C, indicating that lower temperatures offered the enterococci protection from the effects of acidity. Likewise, the enterococci were able to grow at higher salt concentrations at 37 °C compared to the lower temperatures (4, 7 and 10 °C), but the conditions were bacteriostatic and not bacteriocidal for most of the enterococci at all temperatures. Nonetheless, storage at lower temperatures could limit enterococci growth. The
enterococci were able to survive but not grow in butter, Cheddar cheese, cottage cheese, milk powder and yoghurt. The acidic conditions of the yoghurt potentially contributed to the decrease of enterococci in this product during cold storage. Results from this work indicate that combinations of lower temperatures and pH levels and higher salt concentrations do limit the growth of enterococci in dairy products.
CHAPTER 5 - PERSISTENCE OF \textit{ENTEROCOCCUS FAECALIS} IN RAW MILK SILOS IS ASSOCIATED WITH BIOFILM FORMATION AND STREPTOMYCIN RESISTANCE
5.1 Introduction

Enterococci are common intestinal microorganisms that are also widely distributed in the environment, including being present in raw milk, agricultural animals and processed foods (Franz et al., 1999). They have been found to be ubiquitous on dairy farms (Pradhan et al., 2009; Shipp and Dickson, 2012), have been associated with dairy cow mastitis (Gianneechini et al., 2002; Park et al., 2007) and have caused illness in pigs (Cheon and Chae, 1996) and poultry (Abe et al., 2006). In humans, it is known that enterococci have caused infections such as endocarditis for over 100 years (Arias and Murray, 2012). However, nosocomial infections due to enterococci, specifically E. faecalis and E. faecium, have increased in more recent years to become an important health concern in hospitals, with a greater proportion of infections by E. faecium occurring as a result of an increased number of antibiotic-resistant strains (Ogier and Serror, 2008). There is some evidence, through genetic typing of strains, of a link between enterococci strains found in humans and those found in food products (Donabedian et al., 2003; Templer et al., 2008). The concern with antibiotic-resistant strains in the food chain is the potential spread of these, and their genes, within the human digestive tract (Macovei and Zurek, 2007). Inter- and intra-genera transfer of resistance determinants have been reported in the digestive tract using animal models, including transfer from enterococci into other enterococci and L. monocytogenes (Doucet-Populaire et al., 1991; Licht et al., 2002; Moubareck et al., 2003; Lester et al., 2004). The transient acquisition of vancomycin-resistant E. faecium in the human digestive tract, after ingestion of milk inoculated with a commercial poultry-sourced strain (Sørensen et al., 2001), as well as transfer of vancomycin resistance between enterococci (Lester et al., 2006), has been demonstrated in the human gut. Therefore the concern of acquiring antibiotic-resistant bacteria from the food chain is generally acknowledged (Joint Expert Technical Advisory Committee on Antibiotic Resistance, 1999; Barton, 2014).

Although Enterococcus species can be regarded as indicators of faecal contamination, their presence in food products may be from other environmental production and processing
sources (Giraffa, 2003). Prior studies which have investigated the sources of enterococci in sheep and cow milk cheeses were in general agreement that animal faeces were not the source of the enterococci (Gelsomino et al., 2002; Kagkli et al., 2007; Ortigosa et al., 2008) but rather milking equipment (Gelsomino et al., 2002; Kagkli et al., 2007) and the cheesemakers (Ortigosa et al., 2008). It was suggested that niches were present in the milking equipment and bulk storage tanks, providing enterococci strains access to the milk supply (Gelsomino et al., 2002). A factor which may contribute to the persistence of this group, and other bacterial species in the dairy processing environment (Ho et al., 2007; Huck et al., 2007; Di Grigoli et al., 2015), is the potential to form biofilms and adhere to abiotic surfaces (Toledo-Arana et al., 2001), with the further potential for continued re-introduction of these bacteria into milk and subsequently into products.

The repeated use of antimicrobial compounds as disinfectants for the food industry can lead to the development of sanitiser resistance in microorganisms (Aarestrup and Hasman, 2004), which may also contribute to persistence of bacteria in processing environments. Commonly-used disinfectants in the dairy industry include oxidising agents, acid anionic products and quaternary ammonium compounds. Benzalkonium chloride (BC) is a compound in the latter category that has been investigated for its ability to inhibit other microbial species that can be found in food processing environments (Sundheim et al., 1992; Romanova et al., 2006; Magalhães et al., 2016). Resistance to BC can develop through the use of efflux pumps, which can also be associated with multiple drug resistance (Romanova et al., 2006) including resistance to chloramphenicol, ciprofloxacin, erythromycin, streptomycin and tetracycline in certain bacterial species (Danilchanka et al., 2008; Hummel et al., 2007; Pagedar et al., 2011).

A preliminary survey conducted by Food Science Australia in 2001 of enterococci isolated on ECA from raw milk tankers at multiple dairy factories demonstrated that *E. faecalis* was the dominant species detected. Isolates from this survey were used in the assessment of antibiotic resistance (described in Chapter 6) and it was demonstrated that only 50.6 % of
these across the seven species detected were resistant to at least one of tetracycline, streptomycin, erythromycin, chloramphenicol, gentamicin or vancomycin (Chapter 6.3.5). Chapter 2 described a survey of enterococci in raw milk silos from six dairy factories conducted in 2002-2003, which investigated the numbers, prevalence and seasonality of enterococci species over a year. Milk samples contained, on average, $2.48 \log_{10} \text{cfu/mL}$ (Chapter 2.3.4). The dominant species was *E. faecalis*, followed by *E. hirae*, *E. durans* and *E. faecium*, with *E. faecalis* predominant in every season (Chapter 2.3.5). The milk from this survey was further used to investigate persistence of enterococci at one of the factories, as reported in this Chapter.

Enterococci numbers detected in bulk raw milk collected from silos at Factory E over nine months throughout the year were similar to the majority of the other factories studied (Chapter 2.3.4). The purpose of the current work was to investigate whether the occurrence of enterococci at this factory was due to reintroduction or persistence, and if persistence was associated with physiological traits which may influence the persistence of enterococci in the dairy environment (antibiotic or sanitiser resistance, biofilm formation capacity). Milk from the raw milk silos was assessed as a measure of enterococci persistence in the silos. Since the silos are cleaned and sanitised every 1-2 days, finding recurring strains of enterococci would be indicative of strains that have evaded cleaning procedures and persisted in the silos. For the current work, enterococci isolates were collected using enrichment with and without antibiotic selection. As such, this work determined the variation of the antibiotic resistant species present over time, the clonal persistence of the dominant enterococci species at this factory and whether or not there was a correlation between this and physiological characteristics.
5.2 Materials and Methods

5.2.1 Isolation of bulk raw milk isolates

Twenty-five raw milk samples were obtained over a period of nine months from five bulk raw milk storage silos in a dairy factory in the south eastern area of Australia. Samples of 100-200 mL were taken each month from three of the five silos, from June 2002 (winter) until February 2003 (summer), with the exception of September when only one sample was collected. For each sample, 10 mL of raw milk was added to 10 mL of 2XCEB and incubated at 37 °C for 48 h. The turbid culture was mixed 50:50 with 100 % glycerol and frozen at -20 °C until required.

Enterococci were isolated from the samples by dispensing 0.5 mL of the stored, frozen culture into 9.5 mL of CEB or CEB supplemented with each individual antibiotic (chloramphenicol, 32 µg/mL; erythromycin, 8 µg/mL; gentamicin, 16 µg/mL; streptomycin, 1000 µg/mL; tetracycline, 16 µg/mL; vancomycin 6 µg/mL) then incubating at 37 °C for 48 h. Antibiotics were from Sigma (St. Louis, Missouri, USA) except for chloramphenicol and erythromycin, which were from Fluka (Buchs, Switzerland). The resulting cultures were streaked onto two agar plates containing the level of antibiotic corresponding to that in the broth, except for streptomycin plates which contained 2000 µg/mL of antibiotic, which is the level recommended for the detection of high-level streptomycin resistance on agar plates (NCCLS, 2003). Enrichments with no antibiotic, streptomycin or vancomycin were streaked onto BHIA (NCCLS, 2003). All other enrichment cultures were streaked onto Mueller Hinton Agar (MHA, Oxoid) with the appropriate antibiotic. One plate was incubated at 37 °C and the second plate at 45 °C, a selective temperature for enterococci growth (Devriese et al., 2006), for 24 h. Colonies were selected from the plates incubated at 45 °C but if no growth was detected at 45 °C, colonies were taken from the 37 °C plates. Three presumptive enterococci colonies were selected from each plate based on colony morphology and colour (Chapter 2.3.1.1.3), inoculated into 5 mL CEB and incubated at 37 °C.
for 48 h. The cultures were then streaked onto a BHIA slope and onto a BHIA plate to check for purity. The confirmation of isolates as enterococci and speciation was conducted as detailed in Chapter 2.2.6.5 and Chapter 2.2.6.6, respectively.

All strains, including reference cultures, were stored long-term on cryobeads (Chapter 2.2.2) at -70 °C. Working cultures were inoculated into BHIB and grown on BHIA slopes at 37 °C for 20 h for both media, and maintained on the slopes at 4 °C (Chapter 2.2.2).

5.2.2 Reference strains

*E. faecalis* ATCC 29212 (tetracycline resistant) and *E. faecalis* ATCC 51299 (multi-antibiotic resistant, tetracycline sensitive) were used to verify the efficacy of media containing antibiotics. *E. faecium* 64/3 was obtained from Prof. Patrice Courvalin at the Institut Pasteur, France. *E. faecalis* JH2-2 (LMG 19456) was obtained from the Belgium Coordinated Collections of Microorganisms (BCCM), Belgium. *E. faecalis* JH2, the parent strain of JH2-2 (LMG 19456), is reported to be poor at forming biofilms (Kristich et al., 2004). ATCC 29212, *E. faecium* 64/3 and *E. faecalis* JH2-2 were used as controls in the biofilm and sanitiser experiments.

5.2.3 Strain typing

Bulk raw milk *E. faecalis* isolates (n = 218) from the chloramphenicol, erythromycin, gentamicin, streptomycin, tetracycline and no antibiotic enrichments were typed by PFGE following the method of Murray et al. (1990b) as modified by Dahl et al. (1999), using: low-melting temperature agarose (SeaPlaque® Agarose, Bio Whittaker Molecular Applications, Rockland, ME, USA), 1 mg/mL Proteinase K (Sigma-Aldrich, St. Louis, Mo, USA), and 1U/µL Smal (New England Biolabs, Ipswich, MA, USA). A Chef Mapper™ system and model 1000 Mini-chiller (BioRad Laboratories, Hercules, California, USA) were used for the separation of restriction fragments. Restriction profiles were analysed using the Dice coefficient and
Unweighted Pair Group Method with Arithmetic mean clustering with 1 % tolerance and 1 % optimisation settings in GelCompar II, Version 2.5 (Applied Maths, Sint-Martens-Latem, Belgium). Isolates were allotted into PFGE Types where members had 93 % similarity or greater. This percentage similarity was chosen as it allowed biotypes with similar PFGE profiles to be grouped together, with isolates having up to four bands difference. An accepted interpretation of PFGE-generated patterns suggests that one mutational event can be shown in isolates that differ by zero and up to four bands (van Belkum et al., 2007).

One representative isolate from each PFGE Type was selected for determining biofilm formation production and sanitiser susceptibility, taking isolates from as many of the original 25 raw milk samples as possible. When PFGE Types contained isolates obtained both with and without antibiotic selection, one of each was tested. A total of 30 raw milk isolates plus three reference cultures (Chapter 5.2.2) were assessed. The representative isolates in the PFGE Types obtained without antibiotic selection were also assessed for resistance to each of the antibiotics by streaking onto the antibiotic plates.

5.2.4 Assessment of biofilm production

Although it is recognised that there are no standard methods for measuring biofilms, and that laboratory results may not represent what occurs on dairy surfaces, polystyrene is a frequently used surface of choice for in vitro testing (Stepanović et al., 2007; Dunny et al., 2014). Stepanović et al. (2007) made recommendations for the quantification of biofilms in polystyrene microtitre plates, which allows comparison of data across laboratories by using a common surface for binding. The following method is based on a selection of the recommendations described by Stepanović et al. (2007). Isolates were inoculated from BHIA slopes into Tryptone Soya Broth (TSB, Oxoid), grown for 18 h at 37 °C, diluted 1:100 in TSB supplemented with 1 % glucose, dispensed in triplicate in 200 µL aliquots into microtitre plates (96 well, polystyrene, flat bottom) (82.9923.150, Sarstedt Australia Pty Ltd, SA, Australia), covered with a microtitre plate lid and placed inside a plastic container and
incubated (statically) for 24 h ± 15 min at 37 °C. Each well was then aspirated gently with a pipette, washed three times with 200 µL of sterile deionised water and dried in a Class II Biological Hazard safety cabinet for 40 min. The adhered biofilm was fixed with 150 µL of methanol for 20 min and dried in the cabinet for 10 min then stained with 150 µL of 1 % crystal violet for 30 min (Mohamed et al., 2004). Plates were submerged in tap water then water exchanged several times until all free stain was removed. After drying in the cabinet for a minimum of 30 min, bound dye was resolubilised in 150 µL of 95 % ethanol without shaking, and the optical density (OD) was measured at 570 nm using a Spectra Max Plus 384 microtitre plate reader (Molecular Devices, Sunnyvale, California, USA). Three wells containing uninoculated TSB-1 % glucose were used as blanks. Triplicate wells for each isolate and the blank were averaged and the value for the blank was subtracted from the average OD_{570} reading for tests. The entire experiment was replicated three times. The average OD_{570} obtained from triplicate experiments for tests was calculated and the strength of adhesion of biofilms were classified as described by Stepanović et al. (2000) and Mohamed et al. (2004), where isolates were non-adherent (OD < 0.5), weakly adherent (0.5 ≤ OD < 1), moderately adherent (1 ≤ OD < 2) or strongly adherent (OD ≥ 2).

Optical density measurements were statistically analysed using Analysis of Variance with GenStat Release 16.1 (VSN International Ltd., Hemel Hempstead, UK).

5.2.5 Sanitiser susceptibility

A 1 % BC solution was diluted to 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 64 µg/mL in BHIB following the CLSI dilution scheme (NCCLS, 2003). Each concentration and a control consisting of BHIB without BC was dispensed in 225 µL aliquots in triplicate into microtitre plates. Isolates were transferred from BHIA slopes into 10 mL BHIB and incubated for 20 h at 37 °C. The culture was diluted to 10^5-10^6 cfu/mL in BHIB and 25 µL was dispensed into microtitre plates with BHIB and BC. Plates were incubated for 20 h at 37 °C. Wells were visually assessed for turbidity. The MIC was considered to be the first BC concentration in
which there was no visible growth. The BC was obtained from Fluka. Each isolate was assessed in triplicate.

5.3 Results

5.3.1 Enterococcus spp. detected in bulk raw milk

A total of 283 enterococci isolates were obtained from the 25 raw milk samples after selective enrichment in CEB with and without added antibiotics. Four enterococci species, *E. faecalis*, *E. faecium*, *E. hirae* or *E. gallinarum*, were identified (Figure 5.1). The predominant species detected was *E. faecalis* which was isolated without antibiotic selection from 84% of the milk samples. It was the most frequently isolated species following selection with erythromycin, gentamicin and tetracycline and was the only species detected in enrichment broths containing chloramphenicol or streptomycin. Strains of *E. hirae* and *E. faecium* were also found following selection with erythromycin, gentamicin and tetracycline. *E. gallinarum* was only detected when the selective enrichment incorporated vancomycin. No other species were detected on the plates incorporating vancomycin. All of the raw milk samples contained streptomycin-resistant enterococci and 84% of samples contained tetracycline-resistant enterococci, indicating that resistance to these antibiotics is widespread and detectable following selective enrichment.

The detection of enterococci resistant to different antibiotics varied over time and could be related to season with respect to species (Figure 5.1). *E. faecalis* with resistance to erythromycin, streptomycin and tetracycline was detected in all three seasons, while resistance to chloramphenicol and gentamicin were detected in spring and/or summer, the seasons of highest milk production (S. Esposito, Dairy Australia, personal communication, 10-12 May 2005). *E. faecium* was detected solely with tetracycline resistance in summer. In contrast, *E. hirae* with tetracycline resistance was isolated in all three seasons. The vancomycin-resistant *E. gallinarum* isolates were detected only in summer. The greatest
Figure 5.1 Enterococci isolated from bulk raw milk following enrichment in Chromocult Enterococci Broth (CEB) either with or without antibiotic selection. The percentage of isolates of each species detected for each antibiotic selection in (a) all three seasons (b) winter (c) spring (d) summer, where n = the number of isolates from each selection

- E. faecalis, E. faecium, E. hirae, E. gallinarum

Chlor = chloramphenicol, Ery = erythromycin, Gen = gentamicin, Strep = streptomycin, Tet = tetracycline, Van = vancomycin
variation in antibiotic resistance in the enterococci occurred in summer where resistance to all antibiotics tested was observed. The effect of season was not apparent in the incidence of streptomycin-resistant enterococci, as these isolates were detected in all milk samples.

5.3.2 Genetic profiling of *Enterococcus faecalis* isolates

The PFGE profiles of 218 *E. faecalis* isolates were analysed at 93 % similarity, which identified 28 distinct Types (Figure 5.2): 11 Types containing isolates that were obtained without antibiotic selection and 19 Types obtained with antibiotic selection. Two Types contained isolates obtained following enrichment without antibiotic as well as isolates obtained with selection through erythromycin (Type 1) or streptomycin (Type 4), so the number of distinct Types was 28. These two Types represented 35 % of all of the *E. faecalis* isolates screened. When representative isolates in Type 1 were screened for resistance to antibiotics, the isolates in Type 1 were found to be streptomycin resistant. PFGE Types 19, 20, 21 and 23 contained isolates obtained from selection through several different antibiotics (erythromycin, tetracycline or chloramphenicol), which may indicate that the isolates in these PFGE Types are multiply resistant. Results presented in Chapter 6 for *E. faecalis* strains isolated in the summer of 2001 without antibiotic selection from milk tankers showed that tetracycline resistance was widespread (47 % of isolates), high level streptomycin resistance occurred in 32 % of isolates and erythromycin in 12 % whereas chloramphenicol resistance was rare. When multiple resistance occurred, phenotypes of coincident resistance to streptomycin plus tetracycline and streptomycin, tetracycline plus erythromycin dominated and tetracycline/erythromycin resistance was rarer. Single resistance to these antibiotics was seen in less than a third of *E. faecalis* isolates, so this observation is consistent with the potential for isolates in Types with multiple antibiotic selections to be multiply resistant.
Figure 5.2 PFGE Types of *E. faecalis* isolates obtained from bulk raw milk silos following enrichment and selection either with or without specific antibiotics
To facilitate analysis of the incidence of PFGE Types over the duration of the sampling period, the PFGE Types were firstly assorted into groups on the basis of how isolates were originally obtained: without antibiotic selection (Types 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 28) and, for example, streptomycin selection (Types 4, 15, 16, 17, 18 and 27). Within these groups, the Types were categorised as single, multiple, persistent or prevalent based on the frequency of their detection in milk samples and tabulated against their group (Table 5.1). At least half of the Types obtained in the erythromycin and tetracycline selection groups had only single incidences, whereas Types selected without antibiotics were observed to have an even distribution across these categories. In contrast, the majority of Types (83%) for the group selected with streptomycin had multiple, persistent or prevalent incidences, with only one Type containing one isolate showing a single incidence. The most prevalent PFGE Type was Type 4, which contained 58 isolates obtained with either streptomycin or no antibiotic selection. PFGE Type 4 was detected in all three seasons in 24 of 25 raw milk samples, illustrating the wide distribution of this PFGE Type and ability to persist in the milk silos. Without antibiotic selection, this PFGE Type would not have been notable as only five of the 58 isolates were obtained without antibiotic selection. PFGE Type 23, which was prevalent in the milk silos, contained 33 isolates obtained with either erythromycin or tetracycline selection and was detected in nine of the raw milk samples from winter and summer. For PFGE Types that contained more than one isolate, the majority of isolates (64%) came from milk samples from more than one milk silo.

5.3.3 Biofilm production

Table 5.2 presents the number of PFGE Types in each of the biofilm classifications for each antibiotic. The ability of representative raw milk isolates from the 28 PFGE Types of *E. faecalis* to form biofilms is summarized in Figure 5.3 and Table 5.3. Control strain, *E. faecalis* JH2-2, was classified as weakly adherent, as expected, and the remaining two
Table 5.1 Incidence of PFGE Types of *E. faecalis* in raw milk silo samples obtained over nine months

<table>
<thead>
<tr>
<th>Group (Antibiotic selection)</th>
<th>Number of Types(^1) occurring as:</th>
<th>Total number of Types</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>single(^2)</td>
<td>multiple(^3)</td>
</tr>
<tr>
<td>None</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

\(^1\) Incidence is indicated for each antibiotic selection and is categorised as single, multiple, persistent or prevalent based on the frequency of their detection in milk samples and tabulated against their group. When the PFGE Type contained isolates that came from different enrichments, they are listed under each selection type.

\(^2\) a *single* incidence rate is one that occurred only once, in one month, in one silo

\(^3\) a *multiple* incidence rate is one that occurred in more than one silo, but not twice in the same silo

\(^4\) a *persistent* incidence rate is one that occurred in the same silo more than once in the nine months of sampling

\(^5\) a *prevalent* incidence rate is a persistent Type that has occurred in more than one silo in the nine months of sampling
Table 5.2 Classification of strength of biofilm adherence of *E. faecalis* PFGE Types for each antibiotic selection

<table>
<thead>
<tr>
<th>Group (Antibiotic selection)</th>
<th>Number of Types</th>
<th>Number of Types(^1) in each biofilm classification ((%))(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>non-adherent   weak   moderate   strong</td>
</tr>
<tr>
<td>None</td>
<td>11</td>
<td>2 (18)         2 (18)   2 (18)   5 (46)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>2</td>
<td>0              0         2 (100)  0</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>6</td>
<td>0              2 (33)   3 (50)   1 (17)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>1</td>
<td>0              0         0         1 (100)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>6</td>
<td>0              0         0         6 (100)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>9</td>
<td>0              5 (56)   2 (22)   2 (22)</td>
</tr>
</tbody>
</table>

\(^1\) Biofilm classification is indicated for each antibiotic selection. When the PFGE Type contained isolates that came from multiple enrichments, they are listed under each selection type.

\(^2\) Figures in parentheses are the percentage of Types in this category relative to total in the group.
control strains were either non-adherent or moderately adherent: all three were streptomycin sensitive. When a PFGE Type contained members that were originally isolated both with and without antibiotic selection, one isolate obtained with antibiotic selection and one isolate obtained without antibiotic selection were tested. The total number of isolates tested was therefore 30. PFGE Types 19, 20, 21 and 23 contained isolates that came from several different enrichment types, so these PFGE Types are listed multiply in the number of Types in each biofilm classification. For Types where no antibiotic selection was used to obtain isolates, there was a distribution in biofilm-forming capability from non-adherent to strongly adherent (Figure 5.3). In the two PFGE Types which contained isolates with both antibiotic selection and no antibiotic selection, the biofilm classification was the same for both isolates. Apart from isolates at the lower and upper end of the weakly and moderately adherent categories, respectively, isolates in these categories were not significantly different (P > 0.05) in biofilm production (Figure 5.3). Isolates in the strongly adherent category had significantly greater (P < 0.001) biofilm production than the other isolates. The tetracycline-resistant isolates were found in the strongly, moderately and weakly adherent categories, but were more numerous in either the moderately or weakly adherent categories (Figure 5.3). In contrast, all of the streptomycin-selected Types formed strong biofilms. While the only Types with non-adherent isolates consisted of isolates obtained without antibiotic selection, 46 % of the non-antibiotic selected Types formed strong biofilms and 60 % of these were streptomycin-resistant. In the remaining non-antibiotic selected Types, 33 % had streptomycin resistance and 67 % did not have resistance to the six antibiotics assessed. This suggests that there could be a link between streptomycin resistance and increased biofilm production. When the persistence of the PFGE Types was compared with biofilm production, the majority (57 %) of the persistent or prevalent groups showed strong biofilm production.
Figure 5.3 Optical density of biofilm production of *E. faecalis* isolates representing each PFGE Type. PFGE Types containing isolates obtained both with and without antibiotic selection are represented by an isolate obtained with both selections. Antibiotic selections of each isolate are shown: ■ chloramphenicol, ■ erythromycin, ◊ gentamicin, ◊ streptomycin, ◄ tetracycline, □ no antibiotic selection, □ control isolates.
Table 5.3 Summary of the *E. faecalis* PFGE Types showing antibiotic selections and ratings for frequency of detection in milk samples over nine months, biofilm production and susceptibility to the sanitiser benzalkonium chloride

<table>
<thead>
<tr>
<th>PFGE Type</th>
<th>Number in Type</th>
<th>Incidence</th>
<th>Antibiotic selection&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Other resistance&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Biofilm classification</th>
<th>BC MIC&lt;sup&gt;3&lt;/sup&gt; (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>persistent</td>
<td>none (8)/erythromycin (10)</td>
<td>streptomycin</td>
<td>strong</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>single</td>
<td>none</td>
<td>no resistance</td>
<td>strong</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>multiple</td>
<td>none</td>
<td>no resistance</td>
<td>moderate</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>58</td>
<td>prevalent&lt;sup&gt;4&lt;/sup&gt;</td>
<td>none (5)/streptomycin (53)</td>
<td>tetracycline/streptomycin</td>
<td>strong</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>persistent</td>
<td>none</td>
<td>tetracycline</td>
<td>non-adherent</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>persistent</td>
<td>none</td>
<td>no resistance</td>
<td>weak</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>persistent</td>
<td>none</td>
<td>no resistance</td>
<td>moderate</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>single</td>
<td>none</td>
<td>no resistance</td>
<td>strong</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>single</td>
<td>none</td>
<td>no resistance</td>
<td>strong</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>multiple</td>
<td>none</td>
<td>streptomycin</td>
<td>weak</td>
<td>4</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>single</td>
<td>gentamicin</td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>12</td>
<td>6</td>
<td>multiple</td>
<td>tetracycline</td>
<td></td>
<td></td>
<td>4</td>
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<tr>
<td>13</td>
<td>5</td>
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<td>tetracycline</td>
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<td>15</td>
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<td>16</td>
<td>5</td>
<td>multiple</td>
<td>streptomycin</td>
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<td></td>
<td>4</td>
</tr>
<tr>
<td>17</td>
<td>8</td>
<td>persistent</td>
<td>streptomycin</td>
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<td></td>
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<tr>
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<td>2</td>
<td>multiple</td>
<td>streptomycin</td>
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<td></td>
<td>4</td>
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<tr>
<td>21</td>
<td>18</td>
<td>multiple</td>
<td>erythromycin (9)/tetracycline (9)</td>
<td></td>
<td>weak</td>
<td>4</td>
</tr>
<tr>
<td>PFG Type</td>
<td>Number in Type</td>
<td>Incidence</td>
<td>Antibiotic selection</td>
<td>Other resistance</td>
<td>Biofilm classification</td>
<td>BC MIC (µg/mL)</td>
</tr>
<tr>
<td>----------</td>
<td>----------------</td>
<td>-----------</td>
<td>----------------------</td>
<td>------------------</td>
<td>-----------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>22</td>
<td>4</td>
<td>single</td>
<td>tetracycline</td>
<td>strong</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>33</td>
<td>prevalent</td>
<td>erythromycin (22)/tetracycline (11)</td>
<td>weak</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>2</td>
<td>single</td>
<td>erythromycin</td>
<td>moderate</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>3</td>
<td>single</td>
<td>tetracycline</td>
<td>weak</td>
<td>2</td>
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<td>1</td>
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<td>moderate</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>27</td>
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<td>streptomycin</td>
<td>strong</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>1</td>
<td>single</td>
<td>none</td>
<td>streptomycin</td>
<td>non-adherent</td>
<td>4</td>
</tr>
</tbody>
</table>

1 Numbers in brackets are isolate numbers obtained using enrichment without antibiotics or with the listed antibiotic

2 Antibiotic resistance of isolates obtained from enrichments without antibiotic selection (isolates were screened for growth on plates containing chloramphenicol, erythromycin, gentamicin, streptomycin, tetracycline or vancomycin)

3 MIC of benzalkonium chloride

4 When isolates obtained without antibiotic selection were evaluated separately, they were classified as having multiple occurrence
5.3.4 Sanitiser susceptibility

All of the enterococci had MIC values for BC between 2-8 µg/mL. There was very little variability in the susceptibility of the E. faecalis isolates to BC, with 96.4 % of the Types having MIC values of either 2 or 4 µg/mL (Table 5.3). There was no variation in MIC with different antibiotic resistance. All of the control cultures had an MIC of 4 µg/mL, regardless of their different antibiotic resistances and abilities to form biofilms, suggesting that the presence of antibiotic resistance in these isolates is not linked with resistance to the BC sanitiser. Furthermore, the erythromycin-resistant isolate from PFGE Type 23 formed weak biofilms and had an MIC of 2 µg/mL, showing that susceptibility to the sanitiser was not linked to the persistence of the Types or biofilm formation ability.

5.4 Discussion

The experimental strategy in the current study used enrichment broths selective for enterococci containing antibiotics at concentrations that would specifically select antibiotic-resistant enterococci strains, if present and in low numbers. A similar approach was used by Novais et al. (2005) when examining the persistence of antibiotic-resistant enterococci in poultry samples in Portugal. They found that four strains of vancomycin-resistant E. faecium and E. faecalis, with co-resistances to erythromycin, high-level streptomycin and tetracycline persisted in commercial poultry samples for two to three years (Novais et al., 2005). Enrichment in CEB containing antibiotics was used as the primary step in the isolation of the enterococci in the current study in order to detect antibiotic-resistant isolates that may only be present in low numbers in comparison to the other microbiota. The experimental design involved selecting three representative colonies from each antibiotic plate following enrichment and selection, so that the maximum number of isolates in each PFGE Type from each enriched milk sample was three. The observation that large, clonally-related groups of isolates were
obtained following selection through antibiotics suggested that certain PFGE Types were widely dispersed in the raw milk storage silos and that PFGE Types persisted across the sampling period. Consequently, the PFGE Types were examined for their incidence over the duration of the sampling period, where they were categorised as single, multiple, persistent or prevalent based on the frequency of detection in milk samples. The large number of isolates in the antibiotic resistance selected PFGE Types indicates that there was greater uniformity in isolates obtained with antibiotic selection compared with those obtained without selection. As anticipated, there was greater genetic diversity amongst the isolates obtained without antibiotic selection compared to populations selected on the basis of a specific trait, such as high-level resistance to streptomycin.

While *E. faecalis*, *E. faecium* and *E. hirae* were all detected when enriching both with and without antibiotic selection, *E. gallinarum*, a species known as inherently resistant to vancomycin (Leclercq et al., 1992), was the only species detected when the selective enrichment incorporated vancomycin. Vancomycin-resistant *E. faecalis* and *E. faecium* were not detected, indicating a low incidence of resistance to this antibiotic in these species that pose risks in clinical settings (Arias and Murray, 2012). A study in Korea also detected *E. gallinarum* but no vancomycin-resistant *E. faecalis* or *E. faecium* in raw milk (Jung et al., 2007), whereas high levels of vancomycin resistance have been detected in *E. faecalis* and *E. faecium* from raw milk in Botswana and Turkey where it was thought that antibiotic usage in animals may have influenced the occurrence of antibiotic-resistant enterococci (Chingwaru et al., 2003; Citak et al., 2006). In the current study, the most common resistances were to streptomycin and tetracycline. This aligns with the known past use of antimicrobials in lactating cows in Australia, where dihydrostreptomycin and oxytetracycline were among the 10 most frequently dispensed antimicrobials (Dairy Industry Quality Centre, 2001) at the time when the milk used in this study was collected.
Although other studies reported antibiotic resistance of enterococci in raw bovine milk collected milk over a period of time (Chingwaru et al., 2003; Citak et al., 2006; Jung et al., 2007), the seasonal variation of antibiotic resistance in enterococci has not been reported. The present study of bulk raw milk found the detection of erythromycin and tetracycline resistances in Enterococcus spp. collectively were similar between winter and summer seasons. The infrequency of detection of chloramphenicol, gentamicin and vancomycin resistance made determining seasonality uncertain; however, the infrequent detections were all found in summer, which is just after the peak milking season (S. Esposito, Dairy Australia, personal communication, 10-12 May 2005). High-level resistance to streptomycin was widespread in E. faecalis across the seasons, which could be influenced by the frequent use of this antibiotic in Australian dairy herds (Dairy Industry Quality Centre, 2001).

Although there is no standard procedure for classifying BC MIC into sensitive and resistant groups, a study by Sidhu et al. (2001) considered that LAB with MIC levels \( \leq 10 \) µg/mL were considered sensitive, 10-29 µg/mL were considered tolerant, and \( \geq 30 \) µg/mL were considered resistant. Based on this classification, all of the enterococci in the current study were found to be sensitive to BC. This aligns with another study which measured the sensitivity to BC, where E. faecalis (n = 52) had MIC levels of 2-8 µg/mL and E. faecium (n = 78) had MIC levels of 2-16 µg/mL (Aarestrup and Hasman, 2004). In that study, 88.5 % of the E. faecalis had an MIC of 8 µg/mL. Aarestrup and Hasman (2004) also found that the enterococci, which were obtained from food animals, formed one population in their susceptibility to BC and concluded that there had been no development of resistance.

While the ability to form biofilms may assist in persistence, it appears that it is not essential for the persistence of enterococci in the milk silos when this trait was tested here. Antibiotic-sensitive and weakly adherent strains (PFGE Types 6 and 7) were also found to be persistent in the silos. This implies that improper cleaning may enable
populations of bacteria to persist, perhaps due to residual milk in difficult to clean areas. Although it was evident that enterococci strains were persistent in raw milk, within a mixed species environment, they were analysed in pure culture to assess attributes in addition to particular antibiotic resistance. However, biofilm formation in enterococci can be influenced by a number of parameters, including nutritional status of the organism, method used to test biofilm formation (media, nature of material used as the surface) and the presence of other bacteria in the ecosystem (Stanley and Lazazzera, 2004; Stepanović et al., 2007; Fernandes et al., 2015). In the case of the last, biofilm formation in a mixed species environment can be very different as members of the community have an effect on each other. The growth of isolates in mixed species biofilms can result in beneficial, neutral or competitive interactions which may influence biofilm formation (van Merode et al., 2007). In other work, isolates of E. durans and L. lactis subsp. lactis were identified as being effective in controlling the growth of L. monocytogenes in mixed species biofilms possibly due to their bacteriocins (Zhao et al., 2004).

In the current study, there was a strong association of strong biofilm formation with streptomycin resistance, which could have resulted in the persistence of this phenotype in the raw milk silos. Aminoglycoside resistance (gentamicin, amikacin, netilmicin and tobramycin) has been associated with biofilm production in the Gram-positive bacterium Staphylococcus epidermidis (Arciola et al., 2005). Biofilm formation in the Gram-negative bacteria Pseudomonas aeruginosa and E. coli has been shown to be induced by subinhibitory concentrations of aminoglycosides, including streptomycin, due to alterations in the level of cyclic di-guanosine monophosphate, a bacterial secondary messenger that regulates cell surface adhesiveness (Hoffman et al., 2005). There is still emerging evidence on the genetic basis of biofilm formation (Tendolkar et al., 2006; Ballering et al., 2009). Strong biofilm production was specifically associated with gentamicin resistance in E. faecalis (Arciola et al., 2008) and may be due to the up regulation of the adhesin gene efA (Kafil et al., 2016). A relationship to streptomycin
resistance has been reported in Enterococcus spp. (Oliveira et al., 2010). The current work revealed that E. faecalis isolates selected for resistance to streptomycin all had strong biofilm production, with the majority having more than one incidence in the raw milk silos. In particular, one large clonal Type with resistance to streptomycin and strong biofilm production was isolated from all milk samples except one throughout the three seasons. This showed that the persistence of E. faecalis in the raw milk silos was associated with biofilm production and streptomycin resistance.

5.5 Conclusion

Selection with or without antibiotics revealed that there was greater uniformity in PFGE Types amongst those isolates obtained with antibiotic selection. Streptomycin resistance appeared to be linked with the ability of the enterococci to form strong biofilms, whereas erythromycin and tetracycline resistance were more commonly associated with the ability to form moderate or weak biofilms. Strong biofilm production occurred in the majority of persistent or prevalent PFGE Types that also contained streptomycin-resistant isolates, although PFGE Types with members that formed weak and non-adherent biofilms could be persistent as well, indicating that while biofilm production may assist in persistence, it is not essential for the persistence of enterococci in the dairy factory environment. The enterococci formed one population with regards to the susceptibility to benzalkonium chloride and development of resistance to the sanitiser was not apparent. Susceptibility to the sanitiser studied was not linked to persistence or antibiotic resistance. The persistence of enterococci suggests that milk residues remain after cleaning and enterococci are re-introduced from the equipment into subsequent batches of milk. Results indicated a link between the presence of streptomycin resistance, increased biofilm production and persistence of E. faecalis in the milk supply.
CHAPTER 6 - ANTIBIOTIC SUSCEPTIBILITY PATTERNS, VIRULENCE AND MOLECULAR BASIS FOR ANTIBIOTIC RESISTANCE IN ENTEROCOCCI FROM DAIRY SOURCES
6.1 Introduction

Bacterial antibiotic resistance and detection of virulence factors are topics of great interest globally, stimulating many reports and reviews over the last 50 years (Lacey, 1973; Leclercq and Courvalin, 1991; Jett et al., 1994; Holmes and Howden, 2011; Guzman Prieto et al., 2016). Patterns of antibiotic resistance vary with genera and species, location of isolation of exemplars and local health and farming practices (Courvalin, 2005; Wyrsch et al., 2016). Consequently, tracking information for a given antibiotic or virulence factor or drawing inference from prior reports is problematic, particularly if specific traits have received less attention internationally. In Australia, the variation in species occurring in environmental and clinical settings, different local veterinary and clinical antibiotic use and climatic conditions that vary considerably across the continent, warrant local investigation to generate baseline data that may be different to what has been seen elsewhere.

Enterococci have been suspected of causing infections since the beginning of the 20th century (Murray, 1990a), and the use of antibiotics has assisted with their emergence as pathogens (Guzman Prieto et al., 2016). This genera of bacteria has intrinsic resistance to a number of antibiotics, including penicillin due to low-affinity penicillin-binding proteins, cephalosporins, and low levels of clindamycin and aminoglycosides (Murray, 1990a; Swartz, 1994). They can further develop resistance to ampicillin, piperacillin, fluoroquinolones, rifampin, tetracycline, chloramphenicol, macrolides, glycopeptides, which includes vancomycin, and high levels of aminoglycosides (Murray, 1990a; Kayser, 2003). The concern with vancomycin resistance is that is it an antibiotic often reserved for infections due to enterococci with resistance to β-lactams and high levels of aminoglycosides (Bonten, 2001). Resistance to vancomycin can cause severe therapeutic problems in hospitals (Murray, 1997) so that much attention has been given to monitoring patterns and genetic mechanisms of resistance over the last 30 years (Arias and Murray, 2012; Nigo et al., 2014; Guzman Prieto et al., 2016).
In enterococci, the vancomycin-resistant phenotypes that are of medical concern are VanA and VanB, which are conferred by the \textit{vanA} and \textit{vanB} genes (Murray, 1997). These genes may occur in \textit{E. faecalis} and \textit{E. faecium} and are transferable amongst enterococci, whereas the \textit{vanC} genes found in inherently resistant VanC phenotypes are not considered to be transferable (Arthur and Courvalin, 1993). The VanC phenotypes are found in \textit{E. casseliflavus} and \textit{E. gallinarum} (Kak and Chow, 2002) but \textit{vanC} genes have been infrequently found in \textit{E. faecalis} and \textit{E. faecium} (de Garnica et al., 2013; Sun et al., 2014). However, these inherently resistant species are not usually the cause of clinical infection (Murray, 1997; Courvalin, 2006). The species that are of most importance medically are \textit{E. faecium} and, in particular, \textit{E. faecalis} (Franz et al., 1999; Arias and Murray, 2012). However, rare infections have occurred due to \textit{E. casseliflavus} and \textit{E. gallinarum} as well as \textit{E. durans}, \textit{E. hirae}, \textit{E. avium}, \textit{E. raffinosus}, \textit{E. cecorum} and \textit{E. canintestini} (Contreras et al, 2008; Tan et al., 2010; Bello-López et al., 2015; Bourafa et al., 2015; Dicpinigaitis et al., 2015; Labruyère et al., 2015). There is always a possibility that any microorganism with potential for acquiring antibiotic resistance and virulence factors can become pathogenic, so it is worthwhile examining all species that are found in investigations, not just the ones that appear to be immediately relevant.

Investigators have questioned what other properties of enterococci other than antibiotic resistance have led to enterococci being successful pathogens, including adherence, invasion and abscess formation, modulation of host inflammatory responses, and secretion of toxic products (Eaton and Gasson, 2001; Arias and Murray, 2012; Guzman Prieto et al., 2016). Characterised virulence factors include the production of AS, Esp, adhesion of collagen of \textit{E. faecalis} (Ace) and adhesin of collagen of \textit{E. faecium} (Acm), haemolysin, gelatinase, endocarditis and biofilm-associated pili (Epb) and biofilm enhancer in enterococci (Bee), enterococcal polysaccharide antigen (Epa) and glycolipids (Sava et al., 2010; Arias and Murray, 2012). These virulence factors are usually detected via their genes, however haemolysin and gelatinase are additionally sought phenotypically. Several of these are thought to be involved with biofilm formation (Esp, gelatinase, Epb, Bee, Epa and glycolipids) (Arias and Murray, 2012), which is a trait that is investigated in the persistence of bacteria in food environments (Flint et al., 1997; Fernandes et al., 2015). So in addition to potential impact on the severity of illness,
virulence factors may also influence if and how enterococci persist on surfaces (Arciola et al., 2008; Almohamad et al., 2014).

Questions have risen as to whether or not enterococci involved in human disease may have originated in food (Bonten et al., 2001). Very few investigations of enterococci have been conducted on food or food production in Australia. Studies on farms that have been published include incidence of antimicrobial resistant strains in pigs and chickens (Hart et al., 2004; Fard et al., 2011; Obeng et al., 2013) and the presence of enterococci in cattle feedlots (Klein et al., 2010). The presence of enterococci in raw milk in Australia was investigated in Chapter 2, which found that enterococci were widely present in raw milk (Chapter 2.3.4), with \textit{E. faecalis} being the predominant species (Chapter 2.3.5 and Chapter 5.3.1). When the milk was enriched to detect enterococci that were resistant to a selection of antibiotics (chloramphenicol, erythromycin, gentamicin, streptomycin, tetracycline and vancomycin), all of the milk samples contained streptomycin-resistant enterococci (Chapter 5.3.1). Tetracycline-resistant and erythromycin-resistant enterococci were also isolated in the three seasons assessed, winter, spring and summer, but chloramphenicol-resistant and gentamicin-resistant enterococci were found in a limited number of samples (Chapter 5.3.1). Vancomycin resistance was only detected in \textit{E. gallinarum} during the summer (Chapter 5.3.1). Studies on the virulence of enterococci in the dairy environment have not been conducted in Australia. The work presented here looked more broadly at the resistance of enterococci to both medical and veterinary antibiotics and determined the MIC of these antibiotics as well as the genetic basis of resistance for the key antibiotics. The presence of common virulence factors of enterococci from Australian raw milk was also investigated.

A collection of 85 enterococci obtained from a previous study (Chapter 2.2.1) were assessed for the presence of two phenotypic (haemolysin and gelatinase) and two genotypic (AS and Esp) virulence factors. The strains were obtained from raw milk tankers at three factories in different regions of the state of Victoria and were obtained during the summer period in the year prior to the commencement of the raw milk survey (Chapter 2.2.1). These isolates were used since the study of antibiotic resistance
and virulence was conducted in parallel to the year-long survey and a set of isolates obtained from the survey would not have been available until after the completion of the survey, delaying studies on antibiotic resistance for one year. This collection was examined to determine their phenotypic susceptibility to 11 antibiotics used in human medicine (ampicillin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, nitrofurantoin, penicillin G, rifampin, streptomycin, tetracycline and vancomycin) and six veterinary antibiotics (cefoxoxime, cloxacillin, lincomycin, neomycin, novobiocin and oleandomycin). Quality Quarterly (2001) reported on the use of antibiotics for lactating cows in the Australian dairy industry (Table 6.1). All 10 of the antibiotics listed, or their human clinical equivalent, were screened in this study. Several of these, including streptomycin, tetracycline, neomycin, ampicillin and penicillin, are used in both veterinary and human medicine (Quality Quarterly, 2001; Arias et al., 2010; Nigo et al., 2014). A selection of enterococci were assessed for the presence of genes encoding antibiotic resistance for up to five antibiotics (chloramphenicol, erythromycin, streptomycin, tetracycline and vancomycin). The clonality of the isolates was assessed using PFGE. A comparison was made with the prevalence of virulence factors and antibiotic resistance in the Australian isolates with the prevalence of those occurring overseas.

6.2 Materials and Methods

6.2.1 Strains

Wild-type dairy isolates were assessed for the presence of selected virulence factors and resistance to antibiotics. They included 60 E. faecalis, 7 E. casseliflavus, 7 E. gallinarum, 6 E. hirae, 2 E. durans, 1 E. faecium 1 E. malodoratus and 1 species that could not be identified with certainty, which was referred to as Enterococcus sp.

Control strains were used to indicate whether or not phenotypic reactions and Polymerase Chain Reaction (PCR) primers were functioning (Table 6.2). The control strain for both the phenotypic and genotypic virulence factor investigations was isolate MDU4 provided by the Microbiological Diagnostic Unit (MDU, The University of
Table 6.1 Frequency of intra-mammary antimicrobial chemicals dispensed in Australia in 1999 (from Quality Quarterly, 10(2), 2001)

<table>
<thead>
<tr>
<th>Antimicrobial Chemical</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neomycin</td>
<td>21.89</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>16.17</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>12.55</td>
</tr>
<tr>
<td>Dihydrostreptomycin</td>
<td>8.43</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>8.13</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>8.03</td>
</tr>
<tr>
<td>Oleandomycin</td>
<td>8.03</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>7.23</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>5.42</td>
</tr>
<tr>
<td>Penicillin</td>
<td>4.12</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

Table 6.2 Control strains used to confirm phenotypic and genotypic reactions

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Source</th>
<th>Known antibiotic properties¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Chlor</td>
</tr>
<tr>
<td>E. faecalis ATCC 29212</td>
<td>bioMerieux</td>
<td>S</td>
</tr>
<tr>
<td>E. faecalis ATCC 51299</td>
<td>bioMerieux</td>
<td>R</td>
</tr>
<tr>
<td>E. gallinarum ATCC 49573</td>
<td>bioMerieux</td>
<td>-</td>
</tr>
<tr>
<td>E. faecium UW262</td>
<td>Institut Pasteur</td>
<td>-</td>
</tr>
<tr>
<td>E. faecalis LMG 19456</td>
<td>BCCM</td>
<td>S</td>
</tr>
<tr>
<td>E. faecalis LMG 20786¹</td>
<td>BCCM</td>
<td>cat</td>
</tr>
<tr>
<td>E. faecalis LMG 20791¹</td>
<td>BCCM</td>
<td>cat</td>
</tr>
<tr>
<td>E. faecium LMG 20927</td>
<td>BCCM</td>
<td>-</td>
</tr>
<tr>
<td>E. durans LMG 20931</td>
<td>BCCM</td>
<td>-</td>
</tr>
<tr>
<td>E. faecalis MDU4</td>
<td>MDU</td>
<td>-</td>
</tr>
</tbody>
</table>

¹S = Phenotypically susceptible, R = Phenotypically resistant, - = not known at the time of testing; Chlor = chloramphenicol, Ery = erythromycin, Strep = streptomycin, Tet = tetracycline, Van = vancomycin

²These strains are from the same source as LMG 20788 (Irish Cheddar cheese), which is published to have chloramphenicol resistance (Hummel et al., 2007).
Melbourne) which they had been previously confirmed by PCR to be a vanB E. faecalis. The control strains used for phenotypic antibiotic resistance were E. faecalis ATCC 29212 and E. faecalis ATCC 51299 as recommended by The National Committee for Clinical Laboratory Standards (NCCLS, 2003). A selection of enterococi comprising different species and antibiotic resistances from ATCC, BCCM, Institut Pasteur and MDU were used as controls (positive and negative) for the genotypic antibiotic resistance PCRs. All strains were maintained and stored as described in Chapter 2.2.2.

6.2.2 DNA Preparation

Cultures from BHIA slopes (Chapter 2.2.2) were inoculated into BHIB and grown at 37 °C overnight. A 1 mL aliquot was centrifuged at 14000 rpm for 5 min and the supernatant was discarded. To the cell pellet, 200 µl of BioRad InstaGene Matrix (BioRad Corp., Hercules, California, USA) was added and completely resuspended. Samples were heated at 56 °C for 30 min and then at 100 °C for 10 min, with vortexing prior to each step. After the samples were cooled to room temperature, they were vortexed and re-centrifuged. The supernatant (template DNA) was transferred to a clean centrifuge tube and stored at −20 °C until required. Template DNA was diluted 1:10 for use in the PCR reactions.

6.2.3 Isolate identification

6.2.3.1 Confirmation as members of the genus Enterococcus

Members of the collection of 85 isolates were confirmed as Enterococcus with PCR using enterococci-specific tuf primers (Ke et al., 1999). PCR amplifications used 5 µL DNA template, 12.5 µL 2x AmpliTaq Gold DNA mastermix (Applied Biosystems, Foster City, California, USA), 0.25 µL each of forward (Ent 1) and reverse (Ent 2) primers (20 µM) (Table 6.3) and were made up to 25 µL with deionised water. The samples were subjected to 30 cycles of denaturation (95 °C for 30 s), annealing (55 °C for 30 s) and extension (72 °C for 1 min) and a final extension at 72 °C for 7 min. Deionised water was used as a no template control.
6.2.3.2 Confirmation of Enterococcus faecalis

Isolates which were phenotypically determined to be *E. faecalis*, were confirmed as *E. faecalis* genotypically by PCR using primers targeting a region of the 16S rRNA gene (Chapter 6, Table 6.3). The PCR system had been evaluated previously using four *E. faecalis* reference strains, 60 putative *E. faecalis* dairy isolates (Chapter 2.2.1), 10 pak choi putative *E. faecalis* isolates, 19 *Enterococcus* reference isolates other than *E. faecalis*, and 24 other reference and wild-type non-enterococci (King and Wan, unpublished). The PCR preparation included 5 µL DNA template, 10.0 µL 10x AmpliTaq Gold DNA mastermix, 0.1 µL each of the forward and reverse primers and was made up to 20 µL with deionised water. The PCR reaction involved 30 cycles of denaturation (95 °C for 30 s), annealing (66.5 °C for 30 s) and extension (72 °C for 1 min) and a final extension at 72 °C for 10 min.

6.2.4 PCR Method and equipment

PCR amplifications were performed using a GeneAmp® PCR System 9700 (Applied Biosystems). Agarose gels were run at 100-110 V for 60 min using a BioRad Powerpac 300 (Bio Rad Corp). To visualise the PCR products, gels were stained with 0.5 µg/mL ethidium bromide (CLP, San Diego, California, USA) for 30 min, destained in tap water for 30 min and viewed on a UV transilluminator. The images were recorded with a Kodak DC290 camera and Kodak 1D software (Kodak, USA). A 100 bp DNA step ladder (Promega Corporation, Madison, Wisconsin, USA) was used to gage product size.

6.2.5 Pulsed Field Gel Electrophoresis (PFGE) typing

The collection of raw milk enterococci isolates were typed by PFGE following the method of Murray et al. (1990b) as modified by Dahl et al. (1999), using: low-melting temperature agarose (SeaPlaque® Agarose, Bio Whittaker Molecular Applications, Rockland, ME, USA), 1 mg/mL Proteinase K (Sigma-Aldrich, St. Louis, Mo, USA), and 1 U/µL Smal (New England Biolabs, Ipswich, MA, USA). A Chef Mapper™ system and model 1000 Mini-chiller (BioRad Laboratories, Hercules, California, USA) were used for
the separation of restriction fragments. Restriction profiles were analysed using the Dice coefficient and Unweighted Pair Group Method with Arithmetic mean clustering with 1% tolerance and 1% optimisation settings in GelCompar II, Version 2.5 (Applied Maths, Sint-Martens-Latem, Belgium). Isolates were considered to be indistinguishable if they had 97% similarity or greater, as this provided differentiation of strains varying by up to four bands (Chapter 5.2.3).

6.2.6 Phenotypic virulence methods

6.2.6.1 Detection of β-haemolysin
Enterococci were grown in BHIB at 37 °C for 20 h. The isolates were streaked individually onto Columbia agar containing 5% defibrinated horse blood (Oxoid). Plates were incubated at 37 °C for 24-48 h. Zones of clearing around colonies, which is referred to as β-haemolysis, denoted the production of β-haemolysin. The negative control was an uninoculated plate. The positive control was MDU4.

6.2.6.2 Detection of gelatinase
The isolates were grown in BHIB at 37 °C for 20 h. A drop of culture was put onto 10 mL of solidified Nutrient Gelatin (Oxoid) in test tubes. The test tubes were incubated at 37 °C and assessed after 24 h, 48 h and 12 days. To check for liquefaction of the gelatin, the test tubes were submersed in ice water to solidify the melted gelatin. If the media remained liquid, the isolates were able to liquefy the gelatin and were considered positive for gelatinase production. The negative control was an uninoculated tube. The positive control was MDU4.

6.2.7 Genotypic virulence methods

PCR was used to detect the genes for aggregation substance, AS (agg) and enterococcal surface protein, Esp (esp), following the methods of Eaton and Gasson (2001) and Shankar et al. (1999). The forward and reverse primers were TE3 and TE4 (agg) and Esp46 and Esp47 (esp) (Table 6.3). PCR amplifications were performed in 25 μl reaction mixtures using 5 μl DNA template, 12.5 μl 2x AmpliTaq Gold DNA mastermix, 0.8 μl each
of the forward and reverse primers (10 μM), and 5.9 μl deionised water. After an initial
denaturation step at 95 °C for 8 min, the samples were subjected to 30 cycles of
denaturation (94 °C for 45 s), annealing (55 °C for 45 s), and extension (72 °C for 2 min)
and concluded with a final extension at 72 °C for 10 min. Deionised water was used as a
negative control. The agg and esp PCR products were run on 1.5 and 2 % agarose gels,
(Ultrapure Agarose, Life Technologies, Invitrogen, Melbourne, Australia) respectively.

6.2.8 Phenotypic antibiotic resistance methods

Phenotypic sensitivity to antibiotics was assessed by determining the MIC using the
NCCLS (2003) Agar Dilution method. Enterococci isolates were revived from cryogenic
beads by culturing in BHIB for 18-24 h at 37 °C (Chapter 2.2.2). For each isolate, drops of
the BHIB cultures were put into 5 mL of 0.85 % saline to achieve a 0.5 McFarland
Standard. The saline solutions were dispensed into the wells of a template for use with
a Mast multipoint inoculation device (Mast Group Ltd, Merseyside, United Kingdom;
Figure 6.1). Isolates were stamped onto MHA (Amyl Media) containing 10 levels of the
target antibiotics, as well as onto no-antibiotic controls. The plates were stamped in
order from lowest to highest level of antibiotic, to avoid residual carry-over of the
antibiotics. The control plates containing no antibiotic were stamped before and after
the series of 10 plates with antibiotics. Growth on the control plates at the end
confirmed that the isolates were dispensed through the whole series of plates and that
carry over of antibiotics had not occurred. Antibiotic breakpoints (concentration
thresholds for sensitivity and resistance) are listed in Table 6.4. This allows the
distribution of antibiotic susceptibility within a population to be determined. Where
published, the medical break points were used to determine resistance. The MIC90 for
each antibiotic was also determined. MIC90 is the MIC level below which 90 % of the
population of bacteria fall. E. faecalis ATCC 29212 and E. faecalis ATCC 51299 were used
as the control strains for this work, as prescribed by NCCLS (2003). Ampicillin and
chloramphenicol were from Fluka (Buchs, Germany) and ciprofloxacin and gentamicin
were from ICN (Aurora, Ohio, USA). All other antibiotics were from Sigma.
Figure 6.1 Mast multipoint inoculation device used for dispensing multiple bacteria simultaneously onto a series of agar plates containing increasing concentrations of antimicrobial compounds
6.2.9 Genotypic antibiotic resistance methods

Genotypic antibiotic resistance was determined using PCR. PCR amplifications used 5 μL DNA template, 12.5 μL 2x AmpliTaq Gold DNA mastermix and the required forward and reverse primers (20 μM) for the targeted genes (Table 6.3), and were made up to 25 μL with deionised water. All PCR amplifications employed an initial activation and denaturation step at 95 °C for 5 min and a final extension at 72 °C for 7 min. Deionised water was used as a “no template” control. All PCR products were run on 1.5 % agarose gels (Ultrapure Agarose, Life Technologies) at 80 V for 60 min.

6.2.9.1 Chloramphenicol resistance genes
Primers for the cat gene was used to investigate chloramphenicol resistance (Table 6.3). This PCR was done only for isolates phenotypically resistant to chloramphenicol. It used 30 cycles of denaturation (95 °C for 1 min), annealing (49 °C for 1 min) and extension (72 °C for 40 s).

6.2.9.2 Erythromycin resistance genes
Primers for the ermB gene were used to investigate erythromycin resistance (Table 6.3). The PCR employed 35 cycles of denaturation (95 °C for 1 min), annealing (52 °C for 1 min) and extension (72 °C for 1 min). This PCR was performed on a selection of phenotypically sensitive and phenotypically resistant isolates.

6.2.9.3 Streptomycin resistance genes
The enterococci were assessed for the presence of the ant(6)-I streptomycin resistance gene (Table 6.3). PCR amplification involved 30 cycles of denaturation (95 °C for 30 s), annealing (58 °C for 30 s) and extension (72 °C for 30 s). This PCR was performed for all isolates.

6.2.9.4 Tetracycline resistance genes
A multiplex PCR was employed to identify the tetL, tetM and tetS genes, which are responsible for tetracycline resistance in enterococci (Table 6.3). The PCR employed 35
cycles of denaturation (95 °C for 1 min), annealing (55 °C for 1 min) and extension (72 °C for 1.5 min). This PCR was performed for all isolates.

6.2.9.5 Vancomycin resistance genes

The presence of the three main genes responsible for vancomycin resistance in enterococci, \textit{vanA}, \textit{vanB} and \textit{vanC}, was determined in a multiplex PCR reaction (Table 6.3). The reaction employed 30 cycles of denaturation (95 °C for 1 min), annealing (55 °C for 1 min) and extension (72 °C for 1 min) and was done on a selection of isolates that exhibited sensitive and resistant phenotypes.

6.3 Results

6.3.1 Confirmation of the genus \textit{Enterococcus} 

All of the 85 isolates phenotypically identified as \textit{Enterococcus} amplified the \textit{tuf} gene and were considered to be \textit{Enterococcus}. An example of the PCR results is shown in Figure 6.2, giving the anticipated amplicon of 112 bp for all isolates and controls tested.

6.3.2 Confirmation of \textit{Enterococcus faecalis} 

All of the isolates phenotypically identified as \textit{E. faecalis} amplified the required 439 bp amplicon. None of the other species (\textit{E. casseliflavus}, \textit{E. gallinarum}, \textit{E. hirae}, \textit{E. durans}, \textit{E. faecium}, \textit{E. malodoratus} and \textit{Enterococcus} sp.) produced a PCR product of the correct size. The biochemical key (Manero and Blanch, Chapter 2.2.6.6) correctly assigned \textit{E. faecalis} isolates, with no false positive identification to the species.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Use</th>
<th>Gene</th>
<th>Direction</th>
<th>Sequence</th>
<th>Volume per reaction</th>
<th>Amplicon</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ent1</td>
<td>Confirm genus</td>
<td>tuf</td>
<td>forward</td>
<td>TACTGACAAACCATTCTAGATG</td>
<td>0.25 µL</td>
<td>112 bp</td>
<td>Ke et al., 1999</td>
</tr>
<tr>
<td>Ent2</td>
<td>Confirm genus</td>
<td>tuf</td>
<td>reverse</td>
<td>AACTCGTCACCAACCGCAAC</td>
<td>0.25 µL</td>
<td>112 bp</td>
<td>Ke et al., 1999</td>
</tr>
<tr>
<td>EFs 1F</td>
<td>Confirm <em>E. faecalis</em></td>
<td>16S rRNA</td>
<td>forward</td>
<td>CGAGTGGCTGGACTCAATTGG</td>
<td>0.1 µL</td>
<td>439 bp</td>
<td>King and Wan (unpublished)</td>
</tr>
<tr>
<td>EFs 10R</td>
<td>Confirm <em>E. faecalis</em></td>
<td>16S rRNA</td>
<td>reverse</td>
<td>TCAGGGGACTGTTGTTCAACG</td>
<td>0.1 µL</td>
<td>439 bp</td>
<td>King and Wan (unpublished)</td>
</tr>
<tr>
<td>TE3</td>
<td>AS² Virulence</td>
<td>agg</td>
<td>forward</td>
<td>AAGAAAAAGAAATGACCAAC</td>
<td>0.8 µl</td>
<td>1535 bp</td>
<td>Eaton and Gasson (2001)</td>
</tr>
<tr>
<td>TE4</td>
<td>AS Virulence</td>
<td>agg</td>
<td>reverse</td>
<td>AAACGGCAAGACAAACAATTA</td>
<td>0.8 µl</td>
<td>1535 bp</td>
<td>Eaton and Gasson (2001)</td>
</tr>
<tr>
<td>Esp46</td>
<td>Esp³ Virulence</td>
<td>esp</td>
<td>forward</td>
<td>TTACCAAGATGTGGCTTCCCAC</td>
<td>0.8 µl</td>
<td>409, 661 and 936bp</td>
<td>Shankar et al. (1999)</td>
</tr>
<tr>
<td>Esp47</td>
<td>Esp Virulence</td>
<td>esp</td>
<td>reverse</td>
<td>CCAAGTATACTTACATCTTTTG</td>
<td>0.8 µl</td>
<td>409, 661 and 936bp</td>
<td>Shankar et al. (1999)</td>
</tr>
<tr>
<td>Entcatfw</td>
<td>Chlor Resistance</td>
<td>cat</td>
<td>forward</td>
<td>ATG ACT TTT AAT ATT ATT AGG</td>
<td>0.5 µL</td>
<td>648 bp</td>
<td>Hummel et al. (2007)</td>
</tr>
<tr>
<td>Entcatrev</td>
<td>Chlor Resistance</td>
<td>cat</td>
<td>reverse</td>
<td>TCA TTY ACM YTA TSA ATT ATA T</td>
<td>0.5 µL</td>
<td>648 bp</td>
<td>Hummel et al. (2007)</td>
</tr>
<tr>
<td>ErmB f</td>
<td>Ery Resistance</td>
<td>ermB</td>
<td>forward</td>
<td>GAAAGGACTCACAACAAATA</td>
<td>0.25 µL</td>
<td>639 bp</td>
<td>Sutcliffe et al., 1996</td>
</tr>
<tr>
<td>ErmB r</td>
<td>Ery Resistance</td>
<td>ermB</td>
<td>reverse</td>
<td>AGTAACGGTACTTAAATTGTTTAC</td>
<td>0.25 µL</td>
<td>639 bp</td>
<td>Sutcliffe et al., 1996</td>
</tr>
<tr>
<td>Ant(6)-I f</td>
<td>Strep⁴ Resistance</td>
<td>ant(6)</td>
<td>forward</td>
<td>ACTGCGTTAATCAATTGCG</td>
<td>0.625 µL</td>
<td>~600 bp</td>
<td>Swenson et al., 1995</td>
</tr>
<tr>
<td>Ant(6)-I r</td>
<td>Strep Resistance</td>
<td>ant(6)</td>
<td>reverse</td>
<td>GCCCTTCGACCCACTCACC</td>
<td>0.625 µL</td>
<td>~600 bp</td>
<td>Swenson et al., 1995</td>
</tr>
<tr>
<td>TetL f</td>
<td>Tet Resistance</td>
<td>tetL</td>
<td>forward</td>
<td>TGTTAGCTGCTGTCATT</td>
<td>0.625 µL</td>
<td>267 bp</td>
<td>Ng et al., 2001</td>
</tr>
<tr>
<td>TetL r</td>
<td>Tet Resistance</td>
<td>tetL</td>
<td>reverse</td>
<td>GATCCCCAATGTACCG</td>
<td>0.625 µL</td>
<td>267 bp</td>
<td>Ng et al., 2001</td>
</tr>
<tr>
<td>TetM f</td>
<td>Tet Resistance</td>
<td>tetM</td>
<td>forward</td>
<td>GTGCAAAAGGTACACGGAG</td>
<td>0.3125 µL</td>
<td>406 bp</td>
<td>Ng et al., 2001</td>
</tr>
<tr>
<td>TetM r</td>
<td>Tet Resistance</td>
<td>tetM</td>
<td>reverse</td>
<td>CGTAATAATTGCTCACAC</td>
<td>0.3125 µL</td>
<td>406 bp</td>
<td>Ng et al., 2001</td>
</tr>
<tr>
<td>TetS f</td>
<td>Tet Resistance</td>
<td>tetS</td>
<td>forward</td>
<td>CATAGCAAAGCGGTTGACC</td>
<td>0.3125 µL</td>
<td>667 bp</td>
<td>Ng et al., 2001</td>
</tr>
<tr>
<td>TetS r</td>
<td>Tet Resistance</td>
<td>tetS</td>
<td>reverse</td>
<td>ATGTTITTGGAAACCCAGAG</td>
<td>0.3125 µL</td>
<td>667 bp</td>
<td>Ng et al., 2001</td>
</tr>
<tr>
<td>EA1 f</td>
<td>Van Resistance</td>
<td>vanA</td>
<td>forward</td>
<td>GGGAACACGAAATTGC</td>
<td>0.3125 µL</td>
<td>732 bp</td>
<td>Depardieu et al., 2004</td>
</tr>
<tr>
<td>EA2 r</td>
<td>Van Resistance</td>
<td>vanA</td>
<td>reverse</td>
<td>GTACATTCGCGCGTTA</td>
<td>0.5 µL</td>
<td>732 bp</td>
<td>Depardieu et al., 2004</td>
</tr>
<tr>
<td>EB3 f</td>
<td>Van Resistance</td>
<td>vanB</td>
<td>forward</td>
<td>ACGGAATGGAAAGCCGA</td>
<td>0.5 µL</td>
<td>647 bp</td>
<td>Depardieu et al., 2004</td>
</tr>
<tr>
<td>EB4 r</td>
<td>Van Resistance</td>
<td>vanB</td>
<td>reverse</td>
<td>TGCCACCCATTCCGTTC</td>
<td>0.5 µL</td>
<td>647 bp</td>
<td>Depardieu et al., 2004</td>
</tr>
<tr>
<td>ECS f</td>
<td>Van Resistance</td>
<td>vanC1/2</td>
<td>forward</td>
<td>ATGGAATTGTAATCGTAT</td>
<td>0.5 µL</td>
<td>815/827 bp</td>
<td>Depardieu et al., 2004</td>
</tr>
<tr>
<td>ECB r</td>
<td>Van Resistance</td>
<td>vanC1/2</td>
<td>reverse</td>
<td>TAGGGAGGTCGCACGAGTAA</td>
<td>0.5 µL</td>
<td>815/827 bp</td>
<td>Depardieu et al., 2004</td>
</tr>
</tbody>
</table>

¹ AS = Aggregation substance, Esp = Enterococcal surface protein, Chlor = chloramphenicol, Ery = erythromycin, Strep = streptomycin, Tet = tetracycline, Van = vancomycin
Table 6.4 Antibiotic breakpoints used in this study

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Sensitive $^1$</th>
<th>Intermediate</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>$\leq 8$</td>
<td></td>
<td>$\geq 16$</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>$\leq 8$</td>
<td>$16$</td>
<td>$\geq 32$</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>$\leq 1$</td>
<td>$2$</td>
<td>$\geq 4$</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>$\leq 0.5$</td>
<td>$1$-$4$</td>
<td>$\geq 8$</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>$\leq 4$</td>
<td>$8$</td>
<td>$\geq 16$</td>
</tr>
<tr>
<td>Gentamicin HLAR $^2$</td>
<td></td>
<td></td>
<td>$\geq 500$</td>
</tr>
<tr>
<td>Lincomycin $^3$</td>
<td>$\leq 2$</td>
<td>$4$</td>
<td>$\geq 8$</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>$\leq 32$</td>
<td>$64$</td>
<td>$\geq 128$</td>
</tr>
<tr>
<td>Penicillin</td>
<td>$\leq 8$</td>
<td></td>
<td>$\geq 16$</td>
</tr>
<tr>
<td>Rifampin</td>
<td>$\leq 1$</td>
<td>$2$</td>
<td>$\geq 4$</td>
</tr>
<tr>
<td>Streptomycin HLAR</td>
<td></td>
<td></td>
<td>$\geq 2000$</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>$\leq 4$</td>
<td>$8$</td>
<td>$\geq 16$</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>$\leq 4$</td>
<td>$8$-$16$</td>
<td>$\geq 32$</td>
</tr>
</tbody>
</table>

$^1$ Breakpoints are from NCCLS (2003) unless otherwise indicated

$^2$ High level antibiotic resistance

$^3$ NARMS (2014)

Figure 6.2 Example of the tuf PCR to confirm inclusion in the genus Enterococcus

Lanes 1 and 13 - 100 base pair ladder; 2) E. faecalis ATCC 29212; 3) E. faecalis ATCC 51299; 4) E. gallinarum ATCC 29573; 6) E. faecalis LMG 9456; 7) LMG 20786; 8) E. faecalis LMG 20791; 9) E. faecium LMG 20927; 10) E. durans LMG 20931; 11) E. faecalis MDU4; 12) water control
6.3.3 Pulsed Field Gel Electrophoresis

The raw milk tanker isolates were analysed at 97 % similarity (Figure 6.3). Milk from tankers was collected over one summer. Enterococci isolates were assigned a four-digit number, which represented a different milk tanker, plus a letter to differentiate the isolates from the same milk tanker. The majority of the isolates had individual PFGE profiles, with the largest group containing four isolates from three milk tankers delivering to the same factory. In four instances, two E. faecalis isolates with indistinguishable profiles were obtained from the same milk tanker. However, in six instances, isolates with indistinguishable profiles from the same factories were obtained from different milk tankers, primarily with only two isolates in each instance. Two of the E. gallinarum isolates had indistinguishable profiles, while the other isolates with indistinguishable profiles were E. faecalis. Isolates were different between the factories in most cases except in one instance where two E. faecalis isolates, 1969g and 1991r from factories Q and R respectively, were indistinguishable. Most of the non-E. faecalis species clustered with their species, although the pulse types were distinguishable in most instances. The VanC phenotype species, E. casseliflavus and E. gallinarum, also clustered together. There were four isolates with faint profiles that could not be analysed.

6.3.4 Phenotypic and genotypic virulence

6.3.4.1 Raw milk isolates

β-haemolysis was only seen in E. faecalis isolates and in the Enterococcus isolate of uncertain species, representing a detection rate of 8.2 % of all the raw milk enterococci (see Figure 6.4). The trait was detected in 10 % of E. faecalis isolates. A summary of the β-haemolysis results for each strain as well as the results for the other virulence factors is presented in Table 6.5.

Gelatinase activity was only seen in E. faecalis isolates, representing 12.9 % of all of the raw milk enterococci and 18.3 % of E. faecalis isolates.
Figure 6.3 Dendrogram of the raw milk tanker enterococci isolates for which antibiotic resistance and virulence factors were determined (red line indicates 97% similarity)
Table 6.5 Phenotypic antibiotic resistance and virulence factors of raw milk enterococci obtained in 2001

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>Antibiotic resistance</th>
<th>Virulence factors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Amp</td>
<td>Chlor</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>1966b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. faecalis</td>
<td>1967b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. faecalis</td>
<td>1967g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. hirae</td>
<td>1968g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. hirae</td>
<td>1969b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. faecalis</td>
<td>1969g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterococcus sp.</td>
<td>1970g</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>1971b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. faecalis</td>
<td>1971g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. faecalis</td>
<td>1972b</td>
<td></td>
<td>R</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>1972g</td>
<td></td>
<td>R</td>
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<td>E. faecium</td>
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<td>R</td>
</tr>
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<td>1972r2</td>
<td></td>
<td>R</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>1973b</td>
<td></td>
<td>R</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>1973g</td>
<td></td>
<td>R</td>
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<tr>
<td>E. faecalis</td>
<td>1973r1</td>
<td></td>
<td>R</td>
</tr>
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<td>1973r2</td>
<td></td>
<td>R</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>1975b</td>
<td></td>
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<tr>
<td>E. faecalis</td>
<td>1975g</td>
<td></td>
<td>R</td>
</tr>
<tr>
<td>E. gallinarum</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>1975V1.1</td>
<td></td>
<td>R</td>
</tr>
<tr>
<td>E. gallinarum</td>
<td>1975V1.2</td>
<td></td>
<td>R</td>
</tr>
<tr>
<td>Species</td>
<td>Isolate</td>
<td>Antibiotic resistance¹</td>
<td>Virulence factors²</td>
</tr>
<tr>
<td>-----------------</td>
<td>---------</td>
<td>------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amp Chlor Cip Ery Gen Nit Pen Rif Strep Tet Van</td>
<td>agg esp Haem Gel</td>
</tr>
<tr>
<td><em>E. gallinarum</em></td>
<td>1975V2</td>
<td>R R R R</td>
<td>+ - - - -</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>1976b</td>
<td>R</td>
<td>- + - - -</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>1976g</td>
<td>R R R R</td>
<td>- - - - -</td>
</tr>
<tr>
<td><em>E. hirae</em></td>
<td>1976r</td>
<td>R</td>
<td>- - - - -</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>1977g</td>
<td>R R R R</td>
<td>- + - - -</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>1978b</td>
<td>R R</td>
<td>- - - - -</td>
</tr>
<tr>
<td><em>E. gallinarum</em></td>
<td>1978g</td>
<td>R</td>
<td>+ + - - -</td>
</tr>
<tr>
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<td>R</td>
<td>+ - - - -</td>
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1. Antibiotic resistance: Amp = Ampicillin, Chlor = Chloramphenicol, Cip = Ciprofloxacin, Ery = Erythromycin, Gen = Gentamicin, Nit = Nitrofurantoin, Pen = Penicillin, Rif = Rifampicin, Strep = Streptomycin, Tet = Tetracycline, Van = Vancomycin
2. Virulence factors: agg = agg, esp = esp, Haem = Haemagglutination, Gel = Gelatinase
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<th>Virulence factors</th>
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1 R= resistant according to National Committee for Clinical Laboratory Standards (NCCLS, 2003) MIC breakpoints, blank space is not resistant;

Amp = ampicillin, Chlor = chloramphenicol, Cip = ciprofloxacin, Ery = erythromycin, Gen = gentamicin, Nit = nitrofurantoin, Pen = penicillin,
Rif = rifampin, Strep = streptomycin, Tet = tetracycline, Van = vancomycin

2 agg = Aggregation substance gene, esp = Enterococcal Surface Protein gene, Haem = phenotypic β-haemolysis, Gel = phenotypic gelatinase production; + or – indicates virulence factor present or absent.
Isolates showing a PCR product of 1535 bp were deemed positive for the aggregation substance gene *agg*, while those not displaying a band of this size were deemed negative. Of the 85 raw milk isolates, 45.9 % were positive for the *agg* gene. Out of the 39 positive enterococci isolates, 34 of the isolates were *E. faecalis*, although three of the seven *E. gallinarum* isolates, the one *E. malodoratus* isolate and the *Enterococcus* isolate with uncertain identity also had the gene. An example of an *agg* gel, with the culture numbers listed for each lane, is in Figure 6.5.

Products for the *esp* PCR could be various sizes (409, 661 or 936 bp), depending on the number of copies of the repeat region. When the *esp* gene was detected in raw milk isolates, the PCR products were either 409 or 661 bp whereas the control strain, *E. faecalis* ATCC 29212, showed a band at 936 bp (Figure 6.5). The *esp* gene was found in 45.9 % of the raw milk isolates, including 36 *E. faecalis* and 2 *E. gallinarum* isolates, as well as the *Enterococcus* isolate with uncertain identity. Twice as many isolates had the 661 bp product (30 %) compared to the 409 bp product (15 %). Both product sizes were found in the *E. faecalis* and *E. gallinarum* species. The remaining isolate had a product size of 409 bp.

The eight thermoduric strains assessed for heat resistance in Chapter 3 were also screened for the virulence factors. None of the virulence factors were detected in seven of the strains, however the AS gene was detected in *E. faecalis* 2356p1, which also expressed β-haemolysis and gelatinase production.

6.3.4.2 Comparison of virulence factors in European dairy isolates

Virulence factors in *E. faecalis* isolated from bovine milk or cheese in European studies were compared with the raw milk results in this study for *E. faecalis* (Table 6.6). Franz et al. (2001) isolated enterococci from cheese as part of an EU Fair project. Similar to the methods here, they tested for β-haemolysis and gelatinase phenotypically and for AS and Esp by PCR, although they used the *asa1* and *asa373* genes to detect AS. In the Eaton and Gasson (2001) study from the UK, haemolysis and gelatinase were assessed phenotypically as well as by using PCR to detect the genes for AS (*agg*) and Esp (*esp*), haemolysis (*cyl*) and gelatinase (*geLE*) in enterococci isolated from milk and cheese (Eaton and Gasson, 2001).
Figure 6.4 Example of β-haemolysis expressed in *E. faecalis* 1998b

Figure 6.5 Example of PCR gels for (a) *agg* (b) *esp*: Lane 1) 100 bp ladder; 2) 1992b; 3) 1992g; 4) 1993b; 5) 1994g; 6) 1994r; 7) 1995b; 8) 1996g; 9) MDU4; 10) 100 bp ladder; 11) 1997b; 12) 1997g; 13) 1997r; 14) 1998b; 15) 1998g; 16) 1999b; 17) 1999g; 18) water; 19) 100 bp ladder
Table 6.6 Prevalence of virulence factors in *E. faecalis* dairy isolates

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<tr>
<th>Group</th>
<th>Number of <em>E. faecalis</em></th>
<th>Product</th>
<th>Region</th>
<th>agg (%)</th>
<th>esp (%)</th>
<th>Haem&lt;sup&gt;1&lt;/sup&gt; (%)</th>
<th>Gel&lt;sup&gt;1&lt;/sup&gt; (%)</th>
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<td>55</td>
<td>58</td>
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<td>EU</td>
<td>49/32&lt;sup&gt;2&lt;/sup&gt;</td>
<td>36</td>
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<td>49</td>
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<td>75</td>
<td>38</td>
<td>50</td>
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<td>Ireland</td>
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<td>40</td>
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<td>7</td>
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<td>Serbia, Azerbaijan</td>
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<td>86</td>
<td>0</td>
<td>57&lt;sup&gt;3&lt;/sup&gt;</td>
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<td>raw milk</td>
<td>Germany</td>
<td>100&lt;sup&gt;2&lt;/sup&gt;</td>
<td>87</td>
<td>13</td>
<td>13</td>
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</tbody>
</table>

<sup>1</sup> Haem = β-haemolysis, Gel = gelatinase

<sup>2</sup> results for *asa1/asa373*

<sup>3</sup> genetic result for *gelE* only
For haemolysis and gelatinase, only the phenotypic results are presented where possible, as they can be directly compared with this study.

The prevalence of β-haemolysis and gelatinase was considerably less in the Australian isolates compared to the European isolates other than the Valenzuela et al. (2009) study which had no occurrences of β-haemolysis (Table 6.6). However, all of the studies other than Franz et al. (2001) had small sample sizes, which may not be representative of enterococci in dairy samples. The percentage of isolates with *agg* and *esp* were in between the results of the European studies, although Franz et al. (2001) looked for alternate genes.

**6.3.5 Phenotypic and genotypic antibiotic resistance**

**6.3.5.1 Antibiotics with known medical breakpoints**

Raw milk enterococci (85) were assessed for the MIC values of the 11 antibiotics for which breakpoints are known. Not all of the isolates had MIC results since some of the non-*E. faecalis* isolates had insufficient growth on the MHA control plates when diluted to the McFarland Standard used in the NCCLS method. If there was insufficient growth on the control plates, then the test was not valid for those isolates in that instance and results were not used. So the total number of isolates was not always 85 for each antibiotic. This is indicated in the graphs for each antibiotic (Figures 6.6 to 6.16). Of the 11 antibiotics, resistance was detected for ten of them, with no resistance detected against penicillin. The results for each strain are presented in Table 6.5. No resistance to any of the 11 antibiotics was identified in 14.1 % of the isolates, which included six *E. casseliflavus*, three *E. faecalis*, two *E. durans* and one *E. hirae*. However, rifampin resistance was wide spread, present in 83.3 % of the isolates. Of these, 27.1 % had resistance to just to rifampin. Excluding rifampin, 29.4 % had resistance to one antibiotic only. Chloramphenicol, ciprofloxacin and erythromycin resistances were found only in *E. faecalis*. The sole *E. faecium* isolate only had low level resistance to gentamicin. Although resistance to nitrofurantoin was present in 9.9 % of the isolates, this was mainly found in *E. hirae*. Of the six *E. hirae* isolates, five were resistant to nitrofurantoin and the sixth had intermediate resistance. If *E. hirae* was not included amongst the isolates, nitrofurantoin resistance was only present in 3.8 % of the isolates. *E. faecium* and *E. durans*, which are closely related to *E. hirae*, also had
intermediate resistance to nitrofurantoin. After rifampin, the greatest prevalence of antibiotic resistance was to streptomycin and tetracycline which both occurred in *E. faecalis* and *E. gallinarum*. The vancomycin resistance was only found in two isolates of *E. gallinarum*. The thermoduric strains used to assess heat resistance (Chapter 3) were also screened for tetracycline and vancomycin resistance and were found to be sensitive to these antibiotics.

6.3.5.2 Genotypic antibiotic resistance PCR controls

All of the enterococci DNA amplified the *tuf* gene, indicating that the integrity of the DNA was sufficient for PCR. The control strains all amplified the expected antibiotic resistance genes (Figures 6.7c, 6.9c, 6.14c, 6.15c and 6.16c). Both *faecalis* LMG 20786 and *E. faecalis* LMG 20791 amplified the *cat* gene. It was suspected that they might carry this gene but it was unknown. Three of the LMG isolates (*E. faecalis* LMG 20786, *E. faecalis* LMG 20791, *E. faecium* LMG 20927) additionally had the streptomycin *ant(6)* resistance marker. The *E. gallinarum* ATCC 49573 strain had a PCR product at about 667 bp in the tetracycline multiplex PCR, which corresponds with the tetS gene.

6.3.5.3 Ampicillin

The range of MIC values obtained for ampicillin is shown in Figure 6.6a. Three enterococci isolates were recorded as being just resistant to ampicillin (3.7 % of isolates). The ATCC 29212 control strain was sensitive to ampicillin. As the control strain had an MIC one level above the specified range for this antibiotic, it is possible that all of the enterococci tested fall within the sensitive range. However, the MIC90 for these isolates was 8 µg/mL, suggesting that the isolates with MIC values of 16 µg/mL were resistant to ampicillin (Figure 6.6b). Due to their greater prevalence in raw milk in general, *E. faecalis* isolates contributed to the bulk of the MIC results. The lowest MIC levels observed were from species other than *E. faecalis*, although only one of the resistant isolates was *E. faecalis*.

6.3.5.4 Chloramphenicol

Almost the entire range of enterococci MIC values fell below the breakpoint for chloramphenicol resistance (Figure 6.7a). The only isolate that showed resistance was an *E. faecalis* (1.2 % of isolates). More than 98 % of the population was susceptible to
Figure 6.6 Ampicillin resistance of enterococci isolates: (a) MIC values (b) MIC90 values
Figure 6.7 Chloramphenicol resistance of enterococci isolates and controls: (a) MIC values (b) MIC90 values (c) PCR detection of the cat gene: Lane 1) 100 bp ladder; 2) E. faecalis LMG 20786; 3) E. faecalis LMG 20791; 4) E. faecalis 1991b; 5) E. faecalis LMG 19456 negative control; 6) water
chloramphenicol (Figure 6.7b). The single *E. faecalis* isolate (1991b) with phenotypic antibiotic resistance to chloramphenicol amplified the *cat* gene (Figure 6.7c).

6.3.5.5 Ciprofloxacin

Similar to the results for chloramphenicol, the range of MIC levels for the enterococci were mostly under the breakpoint for resistance (Figure 6.8a). One *E. faecalis* isolate showed resistance to ciprofloxacin (1.3 % of isolates). The MIC90 value corresponded well with the breakpoint for ciprofloxacin resistance (4 µg/mL) (Figure 6.8b).

6.3.5.6 Erythromycin

The range of MIC values for erythromycin was bimodal, with the breakpoint for resistance falling in between the two groups of enterococci (Figure 6.9a). One group of enterococci was clearly sensitive to erythromycin while the second group, comprising 9 % of the isolates, was clearly resistant. All of the resistant isolates were *E. faecalis* (Figure 6.9b).

The seven phenotypically erythromycin resistant isolates were all *E. faecalis*. Of these, the *ermB* gene was present in six of them (85.7 %). An example of the PCR is in Figure 6.9c. The gene was also present in *E. gallinarum*. Despite being phenotypically sensitive to erythromycin, the *ermB* gene was present in four of the five *E. gallinarum* isolates assessed. In addition to *ermB*, *E. gallinarum* 1975V2 was phenotypically resistant to streptomycin and tetracycline and had the *ant*(6), *tetL* and *tetM* genes. The co-occurrence of *ermB* and *tetM* was in eight of the ten isolates containing *ermB*, with *E. faecalis* and *E. gallinarum* having four isolates each. On one occasion, *E. faecalis* 1982r contained *ermB* and *tetS*. The other occurrence of *ermB* was in *E. faecalis* 1973r1, which had no other clinical antibiotic resistances.

6.3.5.7 Gentamicin

Only 6 % of the enterococci exhibited standard resistance levels to gentamicin (≥ 16 µg/mL) (Figure 6.10a). Of the five isolates having this level of resistance, four were *E. faecalis* (Figure 6.10b) and the fifth was *E. faecium*. This was the only antibiotic to which the *E. faecium* isolate was resistant. None of the enterococci had gentamicin HLAR (≥ 500 µg/mL).
Figure 6.8 Ciprofloxacin resistance of enterococci isolates: (a) MIC values (b) MIC90 values
Figure 6.9 Erythromycin resistance of enterococci isolates and controls: (a) MIC values (b) MIC90 values (c) PCR detection of the \textit{ermB} gene: Lane 1) 100 bp ladder; 2) \textit{E. faecalis} ATCC 51299; 3) \textit{E. faecalis} ATCC 29212; 4) \textit{E. faecalis} LMG 19456; 5) \textit{E. faecalis} 1972r2; 6) \textit{E. gallinarum} 1973r2; 7) \textit{E. faecalis} 1975b; 8) \textit{E. gallinarum} 1975r; 9) water
Figure 6.10 Gentamicin resistance of enterococci isolates: (a) MIC values (b) MIC90 values
6.3.5.8 Nitrofurantoin

The breakpoint for nitrofurantoin resistance fell in the middle of the range of MIC levels (Figure 6.11a), although the majority of the enterococci were susceptible to the antibiotic. The MIC90 level corresponded exactly with the documented breakpoint level (Figure 6.11b), as 10% of the enterococci were resistant to nitrofurantoin. However, of the eight resistant isolates, only two were *E. faecalis*. Only 3% of *E. faecalis* isolates were resistant to nitrofurantoin. Five of the resistant isolates were *E. hirae*, while other remaining resistant isolate was *E. gallinarum*. There were six *E. hirae* isolates in total, which meant that 83% of the *E. hirae* isolates were resistant, as displayed in Figure 6.11a.

6.3.5.9 Penicillin

All of the enterococci were susceptible to penicillin (Figure 6.12a). Of the top 10 antibiotics dispensed, it was the antibiotic that was least frequently dispensed to lactating cows (Table 6.1). The isolates that were most susceptible to penicillin were all non-*E. faecalis* (Figure 6.12b). MIC90 values were 4 µg/mL.

6.3.5.10 Rifampin

Although the rifampin breakpoint fell mid-range of the antibiotic levels tested, 83% of the enterococci were resistant to rifampin (Figure 6.13a). Eighty eight percent of the *E. faecalis* isolates were resistant, while the remaining isolates had intermediate resistance (2 µg/mL) (Figure 6.13b). Both of the *E. faecalis* control strains (ATCC 29212 and 51299) also had intermediate resistance.

6.3.5.11 Streptomycin

Only the breakpoint for HLAR was listed for streptomycin in the NCCLS procedures (2000). There was a clear distinction between susceptible and resistant isolates (Figure 6.14a), with 27.6% of isolates being resistant. Of the 21 resistant isolates, 19 were *E. faecalis* and two were *E. gallinarum*. Since a high number of the isolates had HLAR, the MIC90 was not applicable (Figure 6.14b), however, the chart does show that the most susceptible isolates were species other than *E. faecalis*. Dihydrostreptomycin was the fourth most commonly dispensed antibiotic to lactating cows (Table 6.1).
Figure 6.11 Nitrofurantoin resistance of enterococci isolates: (a) MIC values (b) MIC90 values
Figure 6.12 Penicillin resistance of enterococci isolates: (a) MIC values (b) MIC90 values
Figure 6.13 Rifampin resistance of enterococci isolates: (a) MIC values (b) MIC90 values
The ant(6) marker for streptomycin resistance was present in all of the streptomycin resistant control strains (Figure 6.14c). As phenotypic streptomycin resistance was so prevalent, all of the isolates were screened for this marker. It was present in five of the total isolates (5.9%). In the 21 isolates with HLAR, this gene was only detected in two (9.5%) of the isolates, both E. gallinarum. The other three isolates with the gene were two E. gallinarum and one E. faecalis. Insufficient growth on MHA by one of the E. gallinarum isolates meant that an MIC was not obtained. It could have had phenotypic resistance but did not grow sufficiently on the test medium. The E. faecalis isolate with ant(6) had an MIC of 1024 µg/mL, one dilution level below the top level at which resistance was determined (2048 µg/mL).

6.3.5.12 Tetracycline

The range of MIC levels for tetracycline was bimodal (Figure 6.15a). Forty percent of the enterococci isolates were resistant to tetracycline, including E. faecalis, E. gallinarum and the Enterococcus sp. isolates. Forty seven percent of the E. faecalis isolates tested were resistant (Figure 6.15b). E. gallinarum was also resistant in general, as five of the seven isolates (71.4%) were resistant. Despite the increased occurrence of resistance compared to streptomycin, tetracycline was less frequently dispensed to cows (Table 6.1).

There were 36 phenotypically tetracycline resistant isolates. The majority of these isolates (97.8%) had at least one of the three tetracycline resistance genes assessed (see Figure 6.15c for example). None of the phenotypically sensitive isolates amplified any of the three genes. The most prevalent gene was tetM, which was present in 91.7% of the resistant isolates, including E. faecalis, E. gallinarum and the Enterococcus sp. It occurred on its own in 80.6% of the isolates, and with tetL in 11% of the resistant isolates. The tetL gene was in 13.9% of the resistant isolates (two E. faecalis, two E. gallinarum and the Enterococcus sp.) and always co-occurred with either tetM or tetS. The tetS gene was detected twice, in E. gallinarum along with tetL, and on its own in E. faecalis 1982r. The two genes tetM and ermB were co-located in the same isolates in eight occasions. The tetS gene was also present in an isolate that had ermB.
Figure 6.14 Streptomycin resistance of enterococci isolates and controls: (a) MIC values (b) MIC90 values (c) PCR detection of the ant(6) gene: Lane 1) 100 bp ladder; 2) E. faecium UW262; 3) E. faecalis ATCC 51299; 4) E. faecalis LMG 20791; 5) E. faecalis LMG 19456; 6) E. faecalis 1966b; 7) E. faecalis 1967b; 8) E. faecalis 1972b; 9) E. faecium 1972r1; 10) E. faecalis 1973r1; 11) E. faecalis 1975g; 12) E. gallinarum 1975V2; 13) E. gallinarum 1978g; 14) E. casseliflavus 1980r; 15) E. casseliflavus 1981r; 16) E. casseliflavus 1987r2; 17) E. faecalis 1991b; 18) E. faecalis 1997b; 19) water; 20) 100bp ladder
Figure 6.15 Tetracycline resistance of enterococci isolates and controls: (a) MIC values (b) MIC90 values (c) PCR detection of the tetL, tetM and tetS genes: Lane 1) 100 bp ladder; 2) E. faecium UW262; 3) E. faecalis LMG 20791; 4) E. gallinarum ATCC 49573; 5) E. faecalis LMG 19456; 6) E. faecalis 1966b; 7) E. faecalis 1967b; 8) E. faecalis 1972b; 9) E. faecium 1972r1; 10) E. faecalis 1973r1; 11) E. faecalis 1975g; 12) E. gallinarum 1975V2; 13) E. gallinarum 1978g; 14) E. casseliflavus 1980r; 15) E. casseliflavus 1981r; 16) E. casseliflavus 1987r2; 17) E. faecalis 1991b; 18) E. faecalis 1997b; 19) water; 20) 100bp ladder
6.3.5.13 Vancomycin

The enterococci isolates were almost completely susceptible to vancomycin (Figure 6.16a), with resistance present in 2.4% of isolates. All of the *E. faecalis* isolates were susceptible (Figure 6.16b). The only isolates that showed vancomycin resistance were two of the inherently resistant VanC phenotype *E. gallinarum* isolates. The other five *E. gallinarum* isolates and three of the *E. casseliflavus* isolates had intermediate resistance, although four of the *E. casseliflavus* isolates were susceptible to vancomycin.

Vancomycin resistance genes were detected in the VanC phenotype species of *E. casseliflavus* (Figure 6.16c). Although *E. gallinarum* 1975V2 had phenotypic vancomycin resistance, it did not amplify the *vanC* gene. The control strain, ATCC 49573 was *E. gallinarum*, and the primers worked for this species. No *vanA* or *vanB* genes were detected in the isolates tested. As none of the non-VanC phenotype species were resistant to vancomycin, the detection of *vanA* and *vanB* genes was not expected.

6.3.5.14 Cefuroxime

The *E. faecalis* ATCC 29212 and ATCC 51299 control strains had MIC values of 8 and 256 µg/mL, respectively. The MIC90 was at 256 µg/mL (Figure 6.17a). There were seven *E. faecalis* and two *E. hirae* isolates at this level or above. Five of the *E. faecalis* isolates had either streptomycin resistance, tetracycline resistance or both. Ampicillin resistance was observed in one other *E. faecalis* isolate.

6.3.5.15 Cloxacillin

The MIC90 for cloxacillin was amongst isolates with MIC values of 32 µg/mL (Figure 6.17b). Both of the *E. faecalis* control strains also had this MIC level. The isolates with MIC values of 64 µg/mL or greater could potentially have resistance to cloxacillin. Isolates in this category included three *E. faecalis*, two *E. hirae* and one *E. casseliflavus*. An *E. hirae* isolate had a value ≥ 256 µg/mL. Co-resistance to other antibiotics was also seen in these isolates, including ampicillin, streptomycin and tetracycline resistances.
Figure 6.16 Vancomycin resistance of enterococci isolates and controls: (a) MIC values (b) MIC90 values (c) PCR detection of the \textit{vanA}, \textit{vanB} and \textit{vanC} genes: Lane 1) 100 bp ladder; 2) \textit{E. faecium} UW262; 3) \textit{E. faecalis} ATCC 51299; 4) \textit{E. gallinarum} ATCC 49573; 5) \textit{E. faecalis} 1966b; 6) \textit{E. faecalis} 1967b; 7) \textit{E. faecalis} 1972b; 8) \textit{E. faecium} 1972r1; 9) \textit{E. faecalis} 1973r1; 10) \textit{E. faecalis} 1975g; 11) \textit{E. gallinarum} 1975V2; 12) \textit{E. gallinarum} 1978g; 13) \textit{E. casseliflavus} 1980r; 14) \textit{E. casseliflavus} 1981r; 15) \textit{E. casseliflavus} 1987r2; 16) \textit{E. faecalis} 1991b; 17) \textit{E. faecalis} 1997b; 18) water; 19) 100bp ladder
6.3.5.16 Lincomycin
The majority of the enterococci MIC values of ≤ 32 µg/mL for lincomycin (Figure 6.17c). *E. faecalis* ATCC 29212 had an MIC value of 32 µg/mL while *E. faecalis* ATCC 51299 had a value exceeding 512 µg/mL. All of the *E. faecalis* and *E. gallinarum* isolates were resistant. The only isolates that fell into the sensitive range were four of the six *E. hirae* isolates.

6.3.5.17 Neomycin
MIC values for neomycin ranged from 2 to ≥ 256 µg/mL, with an MIC90 value of 128 µg/mL (Figure 6.18a). The *E. faecalis* control strains had values of 64 and ≥ 256 µg/mL for ATCC 29212 and ATCC 51299, respectively. Isolates in the ≥ 128 µg/mL range could potentially be considered resistant and included six *E. faecalis* and one each of *E. hirae*, *E. gallinarum* and *E. malodoratus*. This was the only antibiotic to which the *E. malodoratus* isolate may have had resistance. Neomycin was the most frequently dispensed antibiotic to lactating cows in Australia (Table 6.1).

6.3.5.18 Novobiocin
Novobiocin MIC values ranged from 0.5 to ≥ 64 µg/mL (Figure 6.18b). Both of the control strains had values of 16 µg/mL. The MIC90 was amidst the isolates with MIC values of 32 µg/mL. Levels above this could potentially be considered resistant. All of the isolates with MIC values ≥ 32 µg/mL were *E. faecalis*, which were also resistant to streptomycin and tetracycline. Novobiocin is the third most frequently dispensed antibiotic to dairy cows in Australia (Table 6.1).

6.3.5.19 Oleandomycin
The MIC values ranged from 0.25 to ≥ 64 µg/mL for oleandomycin (Figure 6.18c). *E. faecalis* ATCC 29212 had an MIC of 2 µg/mL, which was the value for the majority (62 %) of the enterococci. *E. faecalis* ATCC 51299 had an MIC ≥ 64 µg/mL. The MIC90 level for all of the enterococci was 16 µg/mL, with isolates above this potentially resistant, particularly as most of the isolates were threefold below this. Isolates at this level included six *E. faecalis* and one *E. gallinarum*. Three of these *E. faecalis* isolates were co-resistant to erythromycin, streptomycin and tetracycline.
Figure 6.17 Antibiotic resistance MIC values of enterococci isolates for: (a) cefuroxime (b) cloxacillin (c) lincomycin
Figure 6.18 Antibiotic resistance MIC values of enterococci isolates for: (a) neomycin (b) novobiocin (c) oleandomycin

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6.3.6 Multiple virulence factors and antibiotic resistance

Two thirds of the raw milk enterococci had either none (28 %) or only one (38 %) of the virulence factors tested (Table 6.5). A further 28 % of the isolates had two virulence factors. Multiple virulence factors, in which isolates had three or more factors, were detected in 6 % of the raw milk isolates. When isolates had only one virulence factor, it was twice as likely to be esp than AS. All of the isolates that exhibited β-haemolysis also contained the agg gene for AS and ten out of 11 isolates exhibiting gelatinase contained the agg gene. In contrast, two of the six isolates exhibiting β-haemolysis and two of the 11 isolates exhibiting gelatinase had the esp gene.

Nineteen percent of the raw milk enterococci had resistance to two of the antibiotics tested and 9 % had resistance to three of the antibiotics (Table 6.5). The most common combination was to streptomycin and tetracycline, in 21 % of the isolates. Multiple antibiotic resistance was considered to occur when an isolate was resistant to three or more medical antibiotics, excluding rifampin. None of the isolates had resistance to four or more antibiotics. Thirteen raw milk isolates had either multiple antibiotic resistances and/or multiple virulence factors. None of the isolates with ampicillin or ciprofloxacin resistance possessed multiple antibiotic resistance. The majority of the isolates with multiple antibiotic resistances were E. faecalis, of which 18 % had multiple resistance. The only other species with multiple antibiotic resistance, E. gallinarum, had resistance to vancomycin, to which this species is inherently resistant. All of the isolates with multiple antibiotic resistances were resistant to tetracycline. Three isolates (1997b, 1997g and 2004b) had the erythromycin/streptomycin/tetracycline resistance combination. Of the five isolates with multiple virulence factors (1966b, 1967b, 1972b, 1991r and 1999b), none were resistant to three antibiotics and three showed no antibiotic resistance (Table 6.5).
6.4 Discussion

6.4.1 Enterococcus confirmation

The primers for the enterococci tuf gene are able to detect at least 15 different enterococci species (Ke et al., 1999), so it is reasonable to use this gene as an enterococci DNA control. When the Ent 1 and Ent 2 primers were developed, they were not able to amplify E. solitarius DNA (Ke et al., 1999). However, since that time, it has been proven that E. solitarius is not an Enterococcus but actually belongs to the genus Tetragenococcus (Facklam, 2005). The primers are not completely specific for enterococci, as Abiotrophia species can also be detected, but they do have the potential to assist in the confirmation of enterococci since other Gram-positive bacteria (Gemella, Lactobacillus, Micrococcus, Staphylococcus and Streptococcus) are negative (Ke et al., 1999).

6.4.2 Genetic relationships of the strains

The strains used to assess antibiotic resistance and virulence (Chapter 2.2.1) were obtained in the summer one year prior to the raw milk silo survey (Chapter 2). Results of the survey indicated that enterococci counts were lower in the winter compared to the summer, that there was no difference between the regions but there were differences in enterococci counts between individual factories (Chapter 2.3.4). The strains used to assess antibiotic resistance and virulence were obtained from different factories than the current raw milk survey (Chapter 2.2.5.1). None of the enterococci assessed for antibiotic resistance were genetically similar to the raw milk isolates from the survey, with less than 50 % similarity (results not shown due to excessive size of the dendrogram). It would be reasonable to expect that differences in the enterococci assessed for antibiotic resistance would be factory-specific and not regional, based on the data reported in Chapter 2. However, the prevalence of E. faecalis was greater in the summer period in the raw milk survey and was most prevalent overall (Chapter 2.3.5). The high proportion of E. faecalis found in the isolates from raw milk taken from tankers (70.6 % of total enterococci isolates) is consistent with the prevalence of E. faecalis (61.2-88.5 % of isolates in each factory) in the year-long
raw milk survey (Chapter 2.3.5). The PFGE showed that overall the enterococci assessed for antibiotic resistance were diverse. There were no large clonal groups at the level of similarity examined (97 %), with the largest group of similar isolates containing four *E. faecalis* at the same factory although from three different tankers. This demonstrates that the isolates assessed for antibiotic resistance reflect the makeup of the enterococci population found in the broader one-year survey and represent a wider genetic range of enterococci, providing results that are not based on a small number of clones.

6.4.3 Virulence factors

The incidence of reported β-haemolysis production by enterococci species varies considerably across surveys: several authors observed β-haemolysis in *E. faecalis*, *E. faecium*, *E. avium*, *E. casseliflavus*, *E. durans*, *E. gallinarum* and *E. raffinosus* (Semedo et al., 2003; Gelsomino et al., 2004; Macovei and Zurek, 2007). Giraffa et al. (1995) found in a European study of dairy products that 13 % of *E. faecalis* had this trait, results more aligned with those found for β-haemolysis in the current study, while this trait was not found in isolates from Serbian bovine cheese (Valenzuela et al., 2009). It has been reported that the haemolysin trait appears to be linked with AS production since these two traits are known to be linked on pheromone-responsive plasmids (Franz et al., 2001). Cosentino et al. (2010) noted that the incidence of *agg* and *cyl*, which is the gene involved with β-haemolysis in clinical *E. faecalis*, was explained in part by the clustering of the adhesin and *cyl* genes. In this study, all of the enterococci that showed β-haemolysis had the *agg* gene.

Gelatinase activity has been reported in *E. faecium* and *E. durans*, but at a much lower frequency than in *E. faecalis* (Semedo et al., 2003). A study of human and dairy isolates found that gelatinase was expressed only in *E. faecalis* but not *E. faecium* (Cariolato et al., 2008), perhaps indicating that the phenotypic detection of this trait in species other than *E. faecalis* is random. It has also been observed, however, in *E. hirae*, *E. gallinarum* and *E. casseliflavus* (Lopes et al., 2006; Macovei and Zurek, 2007). Some studies have not looked for gelatinase phenotypically, such as the work by Valenzuela et al. (2009), looking only for one of the original genes determined, *gelE*. That study found a higher proportion of *gelE* compared to other work investigating gelatinase activity. The phenomenon of lower
gelatinase expression compared to the number of strains possessing gelE was investigated in dairy enterococci where it was determined that the complete fsr operon was required for the expression of gelE (Lopes et al., 2006), confirming previous observations of clinical E. faecalis (Qin et al., 2000). However, there were dairy enterococci with the complete fsr operon that did not produce gelatinase (Lopes et al., 2006). Furthermore, significant loss of gelatinase activity occurred with sub-culturing and freezing of isolates in the laboratory, which correlated with loss of genes in the fsr operon (Lopes et al., 2006). Of the 85 enterococci isolates in this study, gelatinase activity was only found among E. faecalis isolates, where 18% of the 60 E. faecalis isolates were positive for the trait. The isolates used in the current study had been frozen at -80 °C for an extended period of time. If substantial loss of the expression of gelatinase activity can occur with freezing, then the results of this study could be an underestimation of the incidence in natural environments. The expression of gelatinase occurred concurrently with the agg gene in the majority of strains in the current work (Chapter 6.3.4, Table 6.5). The agg gene is reported to be involved with the adherence to eukaryotic cells (Eaton and Gasson, 2001). This suggests that in addition to β-haemolysis, agg may also be associated with the production of gelatinase, complementary to the fsr operon, and may be an unexplored reason for lack of gelatinase expression.

In the present study, 55% of the E. faecalis isolated from the raw milk possessed the agg gene. The gene was also detected in 20% of the non-E. faecalis raw milk isolates. The primers for agg were developed from multiple sequence alignments of three E. faecalis sex pheromone plasmids (Eaton and Gasson, 2001) and AS is used to promote the conjugative transfer of sex pheromone plasmids (Archimbaud et al., 2002). It is possible that the gene is naturally present in other enterococci species, or that the gene can be transferred from E. faecalis to other enterococci species.

E. faecalis, E. faecium and E. durans have been reported to have the esp gene (Shankar et al., 1999; Mannu et al., 2003; Semedo et al., 2003). In a study of goat’s milk cheese, though, esp was only found in E. faecalis and not in E. avium, E. hirae, E. malodoratus and E. devreisei also present in the cheese (Martín-Platero et al., 2009). The esp PCR produces three different-sized products and the different lengths of PCR products are possibly due to
repeats of a highly conserved sequence designated as “A repeats” (Shankar et al., 1999). Other researchers have found that *E. faecium* produced bands of greater length compared to *E. faecalis* (Mannu et al., 2003). Two different band lengths were detected in the raw milk *E. faecalis* isolates in this study, indicating a variable number of repeat regions. The number of repeat regions in strains is not correlated with the source of the strains and the significance of them is unknown (Eaton and Gasson, 2002).

### 6.4.4 Comparison of virulence factors in food and clinical enterococci

In addition to comparisons with virulence factors in European food isolates, virulence factors in enterococci from food and clinical sources have been compared by other researchers. In a study comparing food (milk, cheese and meat) and clinical (blood, pus, urine, faeces, hospital) *E. faecalis* and *E. faecium* strains, the clinical isolates of both species generally had a greater incidence of virulence factors (*efaA, gelE, agg, esp*, and cytolysin genes) (Eaton and Gasson, 2001), although the results were not presented individually for each source. Similarly, the majority of enterococci exhibiting the production of gelatinase and β-haemolysis were clinical isolates and not food or environmental, although the food type was not identified in that study (Lindenstrauß et al., 2011). The Italian study of human and dairy isolates, on the other hand, found *esp* and *gelE* were higher in the human *E. faecalis* isolates, whereas *agg* was more abundant in the dairy *E. faecalis* isolates (Cariolato et al., 2008). Another study found *agg, esp* and *cylA* genes to be more prevalent in clinical *E. faecalis* isolates compared to food isolates (vegetables, raw meat, pasteurised milk and dairy products) but that *gelE* was similar between the clinical and food isolates (Medeiros et al., 2014). A Portuguese study comparing ewe’s milk and cheese to clinical enterococci found that β-haemolysis and gelatinase activity was greater in clinical compared to the food isolates (Semedo et al., 2003). As these results were not separated into species, they could not be compared directly with the presence of virulence factors in *E. faecalis* in the current study (Table 6.5). These researchers also detected the *esp* gene in 57 % of food samples and 22 % of clinical samples, in *E. faecalis, E. durans* and *E. faecium*. This contrasts with other work where the *esp* gene was not detected in enterococci from raw ewe’s milk cheese (Mannu et al., 2003). Spanish researchers examined *E. faecalis* and *E. faecium* from meat, dairy and fruit (olives) and did not detect virulence traits (haemolysin, gelatinase, ace,
asa1 and esp) at all (Ben Omar et al., 2004). This is surprising considering that some of the isolates would have been from meat, where the ability to haemolyse red blood cells may provide nutritional benefits to the microorganisms. Overall, the prevalence of these virulence factors was generally greater in the clinical than the food isolates, with few exceptions where agg was greater in food isolates (Cariolato et al., 2008). The gelE was similar between the different sources (Medeiros et al., 2014). This suggests that clinical isolates have either developed these factors as a result of pressures within the medical environment or express these, in clinical environments where they provide a selective advantage during pathogenesis.

6.4.5 Antibiotic resistance

6.4.5.1 Influence of species

In some instances, the presence of resistance could be influenced by species due to potential inherent resistances. For example, ampicillin resistance is usually absent in *E. faecalis* (Sahm, 2005). The most frequently isolated species from the Victorian raw milk was *E. faecalis*, which comprised 71% of the isolates tested for antibiotic MIC levels. It is not surprising, then that the incidence of ampicillin resistance was low. The incidence of vancomycin resistance was also low. Vancomycin resistance was only found in two raw milk isolates of *E. gallinarum*. *E. gallinarum* and *E. casseliflavus* are inherently resistant species (Gholizadeh and Courvalin, 2000). Nonetheless, not all of the inherently resistant species exhibited vancomycin resistance despite the presence of vanC genes, although the majority (8/12) showed intermediate resistance (vancomycin MIC 8-16 µg/mL). These isolates would still be detected in screening for vancomycin-resistant isolates, as the level used for screening is 6 µg/mL (NCCLS, 2003). Rifampin resistance was present in 83% of the *E. faecalis* isolates, with the most sensitive strains belonging to other enterococci species. Although resistance to rifampin mainly occurs due to mutations in the β subunit (Goldstein, 2014), the data here suggest that enterococci may possess a certain degree of inherent resistance to rifampin. *E. faecalis* has been reported to be intrinsically resistant to lincomycin via the lsa gene (Dina et al., 2003). In the current work, all of the *E. faecalis* isolates were resistant (MIC ≥ 8 µg/mL) to lincomycin. The only isolates that were below
this level were four of the six *E. hirae*, an *E. casseliflavus* and the *Enterococcus* sp., suggesting that *E. hirae* at least has inherent susceptibility to lincomycin.

An interesting finding that may not be of concern to the dairy industry but could have clinical relevance was the relationship between *E. hirae* and nitrofurantoin resistance. In the current study, five of the six *E. hirae* isolates (83%) were resistant. This was a much higher prevalence of resistance than the remaining population, where it was present in 3% of *E. faecalis* isolates, for example. This suggests that *E. hirae* may have intrinsic resistance to this antibiotic. The *E. hirae* isolates in this study were also all not closely related to each other (Chapter 6, Figure 6.3), meaning that similarities between them may be due to inherent properties of the species. The nitrofurantoin resistance of *E. hirae* has not been reported. *E. hirae* is not usually associated with human illness but has caused bacteraemia and urinary tract infections (Bourafa et al., 2015; Dicpinigaitis et al., 2015). Nitrofurantoin is one of the antibiotics used to treat enterococci urinary tract infections (Suleyman and Zervos, 2016). As the antibiotic susceptibility of *E. hirae* to nitrofurantoin is currently poorly understood, this could lead to unexpected treatment failures in less common infections due to *E. hirae*. On the other hand, none of the *E. hirae* strains had any of the four virulence factors assessed (Chapter 6.3.4, Table 6.5). Determination of the reason for potential *E. hirae* inherent resistance to nitrofurantoin would be of value, although it is beyond the scope of this study. The potential inherent resistance of *E. hirae* to nitrofurantoin will be used in Chapter 7 as a recipient for antibiotic resistance transfer.

### 6.4.5.2 MIC90

Where the breakpoint is not known, MIC levels above the MIC90 values for a particular antibiotic are considered to be potentially resistant to that antibiotic (Aureli et al., 2003). This approach was used here, particularly for the veterinary antibiotics. Watts et al. (1995) reported MIC90 values of 64 µg/mL and 4 µg/mL for cloxacillin and novobiocin, respectively, for enterococci isolated from the mammary glands of heifers. The current study had an MIC90 one dilution below this for cloxacillin (32 µg/mL) (Chapter 6.3.5.15) but values that were threefold higher for novobiocin (32 µg/mL) (Chapter 6.3.5.18). Cloxacillin and novobiocin were the second and third most commonly used veterinary antibiotics in the year prior to the collection of the isolates used in this study, so there would be increased
potential of the enterococci to develop resistance from greater exposure to these antibiotics. Streptomycin and tetracycline resistances were also observed in isolates with potential resistance to novobiocin and cloxacillin. For several of the antibiotics, the range of MIC values obtained did not result in a clear bimodal distribution between sensitivity and resistance classes, making the determination of resistance less clear. In some instances, such as with oleandomycin and lincomycin, there was a distinct differentiation between the main enterococci population followed by a dramatic increase in the MIC for a few isolates. In such cases, isolates with antibiotic resistance was clear to determine. Although all of the \textit{E. faecalis} isolates were resistant to lincomycin (MIC ≥ 8 µg/mL), there was a subset of isolates, including the ATCC 51299 control strain, that had noticeably greater resistance levels, four to five-fold higher, which suggests that these isolates have a mechanism for HLAR to this antibiotic.

6.4.5.3 \textit{Comparison with overseas phenotypic resistance in dairy products}

Only the results for \textit{E. faecalis} were considered, since only one \textit{E. faecium} isolate was available from the raw milk in the current study, although other species may also carry resistance genes. The presence of antibiotic resistance in European cheese \textit{E. faecalis} (Franz et al., 2001) was greater than the antibiotic resistance found in \textit{E. faecalis} from raw milk in the current study for most of the antibiotics except tetracycline. The studies are similar in that the incidence of resistance was relatively high for the antibiotics streptomycin and tetracycline although erythromycin resistance was more common in the Franz et al. (2001) study. The prevalence of tetracycline resistance in the current study was similar to, although slightly higher than the Franz et al. (2001) study. Neither of the studies found vancomycin resistance in \textit{E. faecalis}. The results differ markedly in that the incidence of chloramphenicol and ciprofloxacin resistance was much higher in the study by Franz et al. (2001). Furthermore, no high level gentamicin or penicillin resistance was found in the current study; however, resistance to these antibiotics was found by Franz et al. (2001).

Other studies on antibiotic resistance in dairy products are not as extensive or as detailed as the Franz et al. (2001) study, with the majority of studies using disc diffusion (end-point studies) or multiple undifferentiated food types, making comparisons difficult. A study of enterococci isolated from Baylough cheese found that 80 % of unique \textit{E. faecalis} strains
were resistant to tetracycline (Gelsomino et al., 2004). *E. faecalis* obtained in the manufacture of Italian mozzarella were not resistant to erythromycin but 42% of the isolates were resistant to tetracycline (Devirgiliis et al., 2010). Franz et al. (2001) also only found vancomycin resistance in one of the 48 *E. faecium* isolates and in none of the 47 *E. faecalis* isolates tested. Vancomycin resistance was not detected in *E. faecalis* from Argentinian bovine cheese (Delpech et al., 2012). In the current study, vancomycin resistance was not detected in the 61 raw milk *E. faecalis* and *E. faecium* isolates or in any of the thirty-eight 10 and 100 g butter, cheddar cheese and milk powder enrichments tested in the dairy product screening (Chapter 2.3.6.2, Chapter 2.3.6.3 and Chapter 2.3.6.4). This will be viewed positively by the Australian dairy industry. However, the surveys were conducted in 2001-2003. The work presented here forms a baseline study that can be used as a reference point for changes over time that may occur with changes in industry practices in antibiotic use and animal stock. Further work could be done to ascertain whether or not the enterococci population has changed in response to potential changes.

6.4.5.4 Genetic mechanisms of resistance

Tetracycline, streptomycin and erythromycin resistance were the most common resistances detected in this work and were investigated further to determine the genetic mechanisms of resistance. Although streptomycin resistance was the most common resistance in this study, the *ant*(6) gene was only detected in 9.5% of the isolates. High level streptomycin resistance can be caused by two known mechanisms, ribosomal mutations or enzyme modification (Nigo et al., 2014). Ribosomal mutations are indicated by streptomycin MIC ≥ 32 000 µg/mL, while enzyme-mediated resistance that confer HLAR is via adenylyltransferase genes *ant*(6′)-Ia and *ant*(3′′)-Ia (Clark et al., 1999; Chow, 2000). The current work used the NCCLS (2003) breakpoints for determining HLAR streptomycin resistance where resistance was ≥ 2000 µg/mL on agar plates. The highest streptomycin concentration tested was 2048 µg/mL and was not extended further. The *ant*(6) gene has been detected in 87.5% of enterococci from cats and dogs (Jackson et al., 2010), 32% of enterococci in pigs (Jackson et al., 2005) but in only just over half of the enterococci from poultry (Jackson et al., 2004), with the basis for resistance in the remaining isolates undetermined. It was proposed that the unidentified resistance mechanism could be ribosomal mutation or the presence of unknown antibiotic resistance genes (Jackson et al.,
The genetic basis for streptomycin resistance is not investigated frequently in food. In a study of farm environment and vegetable samples, the two streptomycin-resistant *E. faecalis* isolated from oats and radish had ant(6) (Ben Said, 2016). Even in studies that have looked for phenotypic streptomycin resistance in food and dairy products (see for example Nawaz et al., 2011; Soares-Santos et al., 2015), the genetic basis for streptomycin resistance has not been reported as frequently as the genes for tetracycline and erythromycin resistance.

The *tetM* gene is widespread amongst both Gram-positive and Gram-negative bacteria (Chopra and Roberts, 2001) and is the most common tetracycline resistance gene in enterococci (Klare et al., 2003). All of the tetracycline-resistant *E. faecalis* isolated from mozzarella harboured the *tetM* gene (Devirgiliis et al., 2010). In enterococci isolated from food, the *tetM* gene was found in 41 of the 43 tetracycline resistant isolates tested and that all strains containing *tetL* also contained *tetM* (Huys et al., 2004). Similar incidences occurred in the present study, except that although *tetL* did not occur in isolation, *tetL* did also occur with *tetS* instead of *tetM*. Also worth noting is that the *tetM* gene is often linked to the *ermB* gene (Chopra and Roberts, 2001). These two genes were both found in eight of the ten erythromycin-resistant enterococci in the current study. On one occasion, an erythromycin-resistant isolate with *ermB* also had *tetS* but not *tetM*. Enterococci are less frequently reported as having *tetS*. These two genes have been found in plasmids in *E. faecalis*, *E. faecium* and *E. gallinarum*, as well as on the chromosome of *E. faecalis* isolated from humans and animals overseas (Novais et al., 2012). In Australian studies, *tetS* was found in 2.7 % of clinical and environmental *E. faecalis* (Rathnayake et al., 2012) but in a study of commensal pig enterococci, it was only detected in *E. gallinarum* and *E. casseliflavus* (Fard et al., 2011). In a study of enterococci from food, mainly cheese, *tetS* was found in 2 % of the isolates as compared to *tetM*, which was in 95 % of the isolates (Huys et al., 2004), which are similar prevalences to the current work. They also found that all 12 of the erythromycin-resistant isolates contained *ermB* (Huys et al., 2004). An investigation of French pasteurised and raw milk cheese found that 96 % and 90 % of erythromycin and tetracycline resistances in the cheese were due to *ermB* and *tetM*, respectively (Jamet et al., 2012) which was similar the work here, suggesting universal presence of these genes in enterococci.
6.4.6 Multiple virulence factors and antibiotic resistance

High level resistance to gentamicin (≥ 500 µg/mL) is considered when screening medical enterococci isolates for antibiotic resistance since it is used to detect synergistic effects with cell-wall acting antibiotics, such as penicillin and vancomycin (NCCLS, 2003). None of the raw milk enterococci isolates had high level gentamicin resistance, or penicillin resistance, so the synergistic effects of the antibiotics should be possible.

A higher incidence of multiple resistance was found in an European study where 49 % of *E. faecalis* had resistance to three or more of the antibiotics listed in Table 6.7 (Franz et al., 2001), contrasting the 8 % of *E. faecalis* with multiple resistance in this study. As in the present study, combined resistance to erythromycin, streptomycin and tetracycline was detected in Europe and South America (Franz et al., 2001; Fracalanzza et al., 2007). The European study found 10 *E. faecalis* isolates (21 %) and one *E. faecium* isolate (2 %) with this pattern (Franz et al., 2001), while this occurred in 3 % of *E. faecalis* isolates investigated here. One isolate of *E. faecalis* with this pattern of resistance was also resistant to chloramphenicol, ciprofloxacin, gentamicin and penicillin (Franz et al., 2001). In contrast to the current work, a German study which investigated raw milk found that 100 % of the 14 *E. faecalis* isolates assessed had resistance to both erythromycin and tetracycline, although streptomycin had not been assessed (Anderson et al., 2016). These isolates also all had the *gelE, asa1* and *efaA* virulence factors, with the majority also having *esp* (Anderson et al., 2016).

Considering the virulence factors assessed in this study (*agg, esp*, haem, gel), none of the food isolates had all four virulence factors. The presence of three factors occurred in five raw milk *E. faecalis* isolates, comprising 6 % of the *E. faecalis* isolates tested. This was similar to but less than European studies, which found between 11-20 % of dairy *E. faecalis* isolates had three of the tested virulence factors, although two of these studies only had a small number of *E. faecalis* isolates from which to compare (Franz et al., 2001; Eaton and Gasson, 2001; Gelsomino et al., 2004). All four of these factors were not found in the study with eight dairy *E. faecalis* isolates (Eaton and Gasson, 2001). In contrast to that and the present study, all four of these virulence factors have been found simultaneously in 11 %
and 20% of dairy *E. faecalis* isolates from the EU (Franz et al., 2001; Gelsomino et al., 2004). Additionally, 17% of dairy *E. faecalis* isolates in the Franz et al. study (2001) had three or more of the virulence factors tested here as well as resistance to three or more antibiotics. None of the Victorian raw milk isolates possessed both multiple virulence factors and three or more antibiotic resistances, although one *E. faecalis* did have both streptomycin and tetracycline resistance together with *agg*, *esp* and β-haemolysis. Hence the occurrence of multiple virulence factors and antibiotic resistance in enterococci in the Australian dairy industry was less than what has been found elsewhere, when the study was undertaken in 2001.

**6.5 Conclusion**

The presence of four virulence factors and antibiotic sensitivity to 11 medical and six veterinary antibiotics was assessed from a collection of raw milk enterococci, of which the predominant species was *E. faecalis*. The isolates were clonally diverse, providing information for a range of isolates more representative of the greater dairy enterococci population. The majority of isolates possessed either none or only one of the virulence factors. Although the incidence of *agg* and *esp* was equivalent, *esp* was in twice as many isolates as *agg* when only one virulence factor was present in an isolate. There was an association between *agg* and the expression of β-haemolysis and gelatinase. Although the relationship of *agg* and β-haemolysis is reported, a potential association between *agg* and gelatinase may be an unexplored reason for lack of gelatinase expression in isolates with *gelE*. The phenotypic virulence factors, β-haemolysis and gelatinase, generally occurred less frequently in Australian isolates compared to those overseas at the time the survey was conducted, although gelatinase may be underestimated due to the previous frozen storage of the isolates. Varied prevalences of the genetic virulence factors *agg* and *esp* amongst studies indicate that the occurrence of these is highly influenced by the particular environments from which the isolates were obtained. Other researchers have found that virulence factors occurred more frequently in clinical isolates compared with food isolates.
Rifampin resistance was extensive in *E. faecalis*, suggesting that this species has a degree of inherent resistance to this antibiotic. Multiple antibiotic resistance to three or more medical antibiotics, excluding rifampin, was only in a small number of isolates, mainly *E. faecalis*, although this also occurred in *E. gallinarum*. The most prevalent antibiotic resistances were to tetracycline followed by streptomycin. Nitrofurantoin resistance was present in almost all of the *E. hirae* isolates, also suggesting potential unknown inherent resistance to this antibiotic in this species. This could have clinical implications if nitrofurantoin was used in the rare occasions in which *E. hirae* is the cause of enterococcal infections. Vancomycin resistance was only found in two intrinsically resistant *E. gallinarum* isolates, which is a species that is not considered to be clinically important. Acquired vancomycin resistance, which is of concern in hospital settings, was not detected in any of the raw milk enterococci or in the enterococci from enriched samples of butter, Cheddar cheese and milk powder (Chapter 2.3.6.2, Chapter 2.3.6.3 and Chapter 2.3.6.4), indicating that resistance to this antibiotic is not commonplace in the variety of enterococci present in the Victorian dairy industry.

The genetic mechanisms for resistance were similar to those detected elsewhere, with *ermB* and *tetM* being the genes responsible for the majority of the erythromycin and tetracycline resistances. These genes were also commonly found in the same raw milk isolates, supporting the theory that these two genes are often linked. Detection of the streptomycin gene *ant*(6) did not correlate well with phenotypic streptomycin resistance, as found in other non-food studies, which suggested that high-level streptomycin resistance may also be caused by ribosomal mutation or the presence of unknown antibiotic resistance mechanisms. There have been several studies where streptomycin resistance has been reported in the absence of being able to detect known plasmid-linked genes. However, the underlying mechanism of resistance is not known and may be related to alteration of the target or active or passive exclusion of the antibiotic. This remains to be explored. Vancomycin resistant genes were only detected in the VanC phenotype species which are inherently resistant to vancomycin. However, as none of the other species had phenotypic resistance to vancomycin, the detection of *vanA* and *vanB* genes was not expected. The prevalence of resistance to various antibiotics in *E. faecalis* was similar or substantially less than in the European work. While virulence factors and antibiotic resistance were detected
in the Victorian isolates, these occurred less frequently overall than in European studies and suggests that the risk of enterococci due to these factors is not as great as in Europe.
CHAPTER 7 - TRANSFER OF ANTIBIOTIC RESISTANCE GENES FROM *ENTEROCOCCUS FAECALIS* TO OTHER ENTEROCOCCI AND *LISTERIA MONOCYTOGENES* ON FILTERS AND IN A MILK BIOFILM MATRIX
7.1 Introduction

Enterococci have a number of intrinsic and acquired antibiotic resistances that pose treatment problems when trying to treat enterococcal infections (Chapter 6.1). They can acquire genetic material via several means, including sex pheromone plasmids, conjugative and non-conjugative plasmids and transposons (Kristich et al., 2014). Sex pheromone plasmids are generally restricted to *E. faecalis* and may confer resistance to antibiotics such as chloramphenicol, erythromycin, vancomycin, tetracycline, penicillin, gentamicin, streptomycin, kanamycin and tobramycin (Franz et al., 1999; Lim et al., 2006; Sedgley et al., 2009). Conjugative plasmids transfer at a lower frequency compared to sex pheromone plasmids, but they have a broader recipient range (Franz et al., 1999; Kristich et al., 2014). For example, the plasmid pAMβ1, which confers resistance to erythromycin, has been transferred from *E. faecalis* into lactococci, lactobacilli, *Bacillus spp.*, and *S. aureus* (Clewell, 1990; Franz et al., 1999). Transposons in the transposon integrase gene family Tn916-Tn1545 are present in numerous antibiotic-resistant enterococci (Huys et al., 2004; Cauwerts et al., 2007; Ramos et al., 2012). The Tn1545 is associated with the *tetM* resistance gene and the *ermAM* resistance gene (Rice, 1998), now known as *ermB* (Roberts et al., 1999). Tetracycline and erythromycin resistance co-occurred in *E. faecalis* isolated from raw milk, (Chapter 6.3.4), with the *tetM* and *ermB* genes detected in the same isolates (Chapter 6.3.4.6), and further work could elucidate if this may be due to the presence of the Tn1545 transposon.

As mentioned in Chapter 1.3.2, the ability of enterococci to exchange genetic material has been studied for years (Jones et al., 1987; Noble et al., 1992; Perreten et al., 1997; Clewell, 2011). Much of the focus has been in regards to the two species of most interest medically, *E. faecalis* and *E. faecium* (Clewell et al., 2014). However, other species have occasionally been responsible for human infections including *E. hirae*, *E. durans*, *E. gallinarum*, *E. cecorum*, *E. avium* *E. raffinosus*, *E. casseliflavus* and *E. canintestini* (Canalejo et al., 2008; Antonello et al., 2010; Tan et al., 2010; Kenzaka et al., 2013; Berenger et al., 2015; Shanmugakrishnan et al., 2015). As with *E. faecalis* and
*E. faecium* (Arias and Murray, 2012), antibiotic resistances in these species would presumably also make infections from these species more difficult to treat. Therefore, the transfer of antibiotic resistance determinants to these species is of interest, in addition to transfer between *E. faecalis* and *E. faecium*. Experiments elsewhere have included the transfer of *ermB* and *vanA* from *E. durans* to *E. faecium* (Vignaroli et al., 2011), *ermB* from *E. durans* to *E. faecalis* (Thumu and Halami, 2014), *cat* from *E. faecalis* to *E. italicus* (Borgo et al., 2009) and *tetS* from *E. italicus* to *E. faecalis* (Zago et al., 2010).

Most antibiotic resistance transfer experiments begin with looking at transfer using filter mating. Since transfer of resistances have been demonstrated in the laboratory, it is valuable to assess the possibility of transfer in applicable in vivo situations. This has been investigated infrequently in food industry applications but a few studies have looked at antibiotic resistance transfer from *E. faecalis* in sausage and cheese fermentations (Cocconcelli et al., 2003; Gazzola et al., 2012), on pork meat slices and dry sausage (Rizzotti et al., 2009) and in milk (Borgo et al., 2009). Another study demonstrated transfer of *ermB* from *Leuconostoc mesenteroides* to *E. faecalis* on Monte Veronese cheese slices and found that the transfer frequency was higher in the food matrix compared to filter mating (Florez et al., 2016). On the other hand, the in vivo mating experiments of Borgo et al. (2009) in milk and Rizzotti et al. (2009) on meat slices had similar and lower transfer frequencies than those obtained using filter mating. This suggests that the ability of enterococci to transfer genes in natural food matrices will be influenced by differences in the strains and the properties of the food matrices.

The purpose of the current study was to determine if the most commonly encountered antibiotic resistances found in Australian raw milk enterococci could transfer, either singly or together, to other *Enterococcus* species and to *L. monocytogenes* both in filter mating and in a milk biofilm. *L. monocytogenes* is a human pathogen causing potentially fatal illness in susceptible individuals and is found in raw milk or as a post-pasteurisation contaminant in pasteurised milk (Quigley et al., 2013). *E. faecalis* was the predominant species isolated in the raw milk survey (Chapter 2.3.5) as well as in the set of isolates used for the assessment for antibiotic resistance (Chapter 6.2.1). The most prevalent antibiotic resistances were to tetracycline and streptomycin, followed by erythromycin.
(Chapter 6.3.4), which were also concurrently detected in three *E. faecalis* isolates (Chapter 6.3.5). These resistances were also seen in the greatest number of bulk raw milk samples that were enriched for antibiotic-resistant enterococci (Chapter 5.3.1). Two *E. faecalis* isolates with slightly different antibiotic resistance profiles and genetic mechanisms were selected as donor isolates. *E. faecalis* 1991b was resistant to chloramphenicol (*cat*), erythromycin (*ermB*) and tetracycline (*tetL* and *tetM*) and had the *ant*(6) gene even though it was phenotypically sensitive to streptomycin (Chapter 6.3.4). *E. faecalis* 2004b was resistant to erythromycin (*ermB*), streptomycin and tetracycline (*tetM*) (Chapter 6.3.4), although the resistance mechanism for streptomycin resistance was unknown. In addition to the *E. faecalis* JH2-2 and *E. faecium* 64/3 isolates used as recipients in overseas antibiotic resistance transfer experiments (based on their resistance to rifampin and fusidic acid), an *E. hirae* isolate (1981g) selected from the current study (Chapter 6) and an Australian *L. monocytogenes* dairy isolate were used as alternative recipient isolates. The apparent inherent resistance of *E. hirae* to nitrofurantoin (Chapter 6.4.5.1), along with induced rifampin resistance, were used for antibiotic resistance counter selection in the detection of *E. hirae* transconjugants.

### 7.2 Materials and Methods

#### 7.2.1 Strains

**7.2.1.1 Modification of Enterococcus hirae as a recipient strain**

Like the other *E. hirae* assessed in the current studies, *E. hirae* 1981g was resistant to nitrofurantoin at 256 µg/mL on MHA using the NCCLS (2003) procedure (Chapter 6.3.4.8). This isolate, along with the antibiotic-resistant donor isolates *E. faecalis* 1991b, *E. faecalis* 2004b and *E. faecalis* LMG 20790 (Table 7.1), were grown in BHIB (21 h at 37 °C) with three levels of nitrofurantoin, 50, 100 and 150 µg/mL, to determine the lowest level of nitrofurantoin that inhibited growth of the donor isolates. None of the donor isolates grew in the broths and the lowest level of nitrofurantoin (50 µg/mL) was selected.
To obtain rifampin resistant mutants, *E. hirae* 1981g was spread (0.1 mL) onto BHIA containing 50 μg/mL of nitrofurantoin plus 50 μg/mL of rifampin. Colonies were picked from these selective plates after 24 h at 37 °C and sub-cultured three times on this medium before storing the most vigorous mutant, designated 1981grf3, on cryobeads (Chapter 2.2.2).

7.2.1.2 Donors

*E. faecalis* 1991b and 2004b were selected from Chapter 5 as donors due to the presence of multiple antibiotic resistances (Table 7.1). *E. faecalis* LMG 20790 was purchased from the BCCM. It was previously used in antibiotic resistance transfer experiments and was used as a control to ascertain that antibiotic resistance transfer occurred in this laboratory as published previously (Huys et al., 2004).

7.2.1.3 Recipients

*E. faecalis* JH2-2 was purchased from BCCM under the culture number LMG 19456 (Table 7.1) (Huys et al., 2004) and *E. faecium* strain 64/3 (Moubareck et al., 2003) was obtained from Pr. Patrice Courvalin at the Institut Pasteur, Paris, France. *E. hirae* 1981grf3 was developed as described above (Chapter 7.2.1.1) for use as a recipient of antibiotic resistance transfer. *L. monocytogenes* E17 was obtained from the CSIRO culture collection as an Australian dairy *Listeria* antibiotic resistance transfer recipient.

7.2.2 Filter mating

7.2.2.1 Determination of MIC for antibiotics used in mating procedures

The MIC of the primary antibiotics used in the mating experiments were measured on BHIA on late exponential phase *Enterococcus* cultures to determine the ability of donors and recipients to grow in conditions similar to those used in the mating experiments. The cultures assessed were the *E. faecalis* and *E. faecium* strains listed in Table 7.1 as well as the initial *E. hirae* 1981g parent culture.
Table 7.1 Strains used as donors and recipients of antibiotic resistance transfer

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Antibiotic resistance phenotype (gene)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Donors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> 1991b</td>
<td>Australian raw milk</td>
<td>chloramphenicol (<em>cat</em>) erythromycin (<em>ermB</em>) streptomycin (<em>ant(6)-I</em>) tetracycline (<em>tetL, tetM</em>)</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. faecalis</em> 2004b</td>
<td>Australian raw milk</td>
<td>Erythromycin (<em>ermB</em>) streptomycin (unknown) tetracycline (<em>tetM</em>)</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. faecalis</em> LMG 20790</td>
<td>Irish cheddar cheese</td>
<td>Erythromycin (<em>ermB</em>) Tetracycline (<em>tetM</em>)</td>
<td>Huys et al., 2004</td>
</tr>
<tr>
<td><strong>Recipients</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. faecium</em> 64/3</td>
<td>Derived human faecal isolate</td>
<td>fusidic acid rifampin</td>
<td>From Klare et al. (1992), used in Moubareck et al., 2003</td>
</tr>
<tr>
<td><em>E. hirae</em> 1981grf3</td>
<td>Derived Australian raw milk</td>
<td>nitrofurantoin rifampin</td>
<td>This study</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> E17</td>
<td>Australian yoghurt factory drain</td>
<td>susceptible to antibiotics used for gene transfer</td>
<td>CSIRO Werribee Collection</td>
</tr>
</tbody>
</table>

Table 7.2 Antibiotics and dilution ranges used to determine MIC on BHIA

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentrations assessed (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin</td>
<td>4, 8 and 16</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>16, 32, 64, 96 and 128</td>
</tr>
<tr>
<td>Rifampin</td>
<td>16, 32, 64, 128, 192 and 256</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>125, 250, 500, 1000 and 2000</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>16, 32, 50 and 64</td>
</tr>
</tbody>
</table>
Isolates were inoculated into 10 mL BHIB from BHIA slopes and grown at 37 °C for 20 h, then cultures diluted 10-fold using 0.1 % Bacteriological Peptone and applied to BHIA, or BHIA containing a range of antibiotics (Table 7.2), using a Mast multipoint inoculation device (Chapter 6.2.8). Fusidic acid was obtained from Sigma. The range of antibiotic concentrations tested encompassed concentrations used in published antibiotic resistance transfer methods (Moubareck et al., 2003; Huys et al., 2004). Plates were incubated at 37 °C for 20 h. Chloramphenicol was used at 10 or 20 µg/mL, as previously determined.

7.2.2.2 Filter mating procedure
Filter mating was based on the method used by Huys et al. (2004). Donors and recipients were grown for 20 h at 37 °C in BHIB, diluted to ~ 10^5 cfu/mL in BHIB and grown further for 5 h at 37 °C. Equal proportions of donor and recipient were mixed together and then 2 mL filtered through a HAWP2500 filter (Millipore, Ireland). The filter was rinsed with 100 mL of sterile deionised water, as recommended by Sasaki et al. (1988). Filters were placed right side up on BHIA and incubated for 20 h at 37 °C. Cells were recovered by vortexing the filter in 2.5 mL of 0.1 % Bacteriological Peptone. Filter mating was replicated twice.

7.2.2.3 Isolation of transconjugants
Recovered enterococci cells were serially diluted and plated onto BHIA containing 25 µg/mL fusidic acid and 50 µg/mL rifampin, to select for the E. faecalis and E. faecium recipients, or 50 µg/mL rifampin plus 50 µg/mL nitrofurantoin, for the E. hirae recipient, plus a third antibiotic of interest. These consisted of chloramphenicol (10 µg/mL), erythromycin (4 µg/mL), streptomycin (128 µg/mL) and tetracycline (10 µg/mL) for the E. faecium recipient. For the E. faecalis and E. hirae recipients, the erythromycin and tetracycline concentrations were as indicated for E. faecium, but the chloramphenicol and streptomycin concentrations were 20 µg/mL and 500 µg/mL, respectively. A BHIA plate without antibiotics was used to assess total cell viability. Plates were incubated at 37 °C for 24 h. Up to 100 presumptive transconjugant colonies were selected from each selective agar and were replicated onto the other triple selection media using velveteine to determine transfer of multiple resistance.
L. monocytogenes E17 was mated solely with E. faecalis 1991b, with Listeria transconjugants detected on Oxford Agar (Oxoid) containing chloramphenicol (10 µg/mL), erythromycin (4 µg/mL), streptomycin (500 µg/mL) or tetracycline (10 µg/mL). Colonies were selected and replicated as for the enterococci using Oxford Agar.

7.2.2.4 Differentiation of Enterococcus transconjugants

Carbohydrate fermentations were used to differentiate whether presumptive transconjugants were recipient strains which acquired antibiotic resistance from the donors, or if the donor strains mutated or acquired resistance from recipients. In order to do this, the E. faecalis and E. faecium strains were first characterised using API Rapid ID 32 Strep (bioMérieux, Marcy-l’Etoile, France) further characterise and determine any additional differences between the isolates that were not already known through the carbohydrate fermentation scheme of Manero and Blanch (1999). Properties of E. hirae were as described in Manero and Blanch (1999).

Strains were inoculated into BHIB from BHIA slopes and grown at 37 °C for 20 h. Broths were streaked onto BHIA and grown at 37 °C for 20 h. Following the manufacturer’s directions, colonies were used to inoculate API Suspension Medium to a turbidity of McFarland Standard 4. API strips were inoculated with the suspension and incubated at 37 °C for 4 h. Positive and negative reactions were recorded as indicated in the instructions.

The carbohydrates selected for differentiating the strains were α-cyclodextrin, arabinose and mannitol (Sigma). These were added at 0.5 % to Phenol Red Broth with agar plates (Oxoid). When the transconjugants were replicated onto each of the triple selection media, they were additionally replicated onto the carbohydrate plates to confirm that the E. faecalis, E. faecium or E. hirae recipient strains were not the donor strains. Following the carbohydrate utilisations listed in Table 7.3, arabinose was used to determine E. faecium tranconjugants (isolates positive), mannitol was used to determine E. hirae tranconjugants (isolates negative) and α-cyclodextrin was used to
determine *E. faecalis* JH2-2 transconjugants (isolates negative). A positive and negative culture was spotted onto each plate.

### 7.2.2.5 Confirmation of gene transfer

Confirmation of gene transfer was determined in up to 10 of the presumptive transconjugants from the initial selective agar plate using PCR. DNA was harvested from the presumptive transconjugants by growing them in BHIB at 37 °C for 18-22 h, then boiling 1 mL of the broth for 10 min in a microcentrifuge tube (Convert et al., 2005) and cooling to room temperature. The cooled broth was used as the DNA template. PCR reactions were conducted according to Hummel et al. (2007) for *cat* (chloramphenicol), Sutcliffe et al. (1996) for *ermB* (erythromycin), Swenson et al. (1995) for *ant*(6)-I (streptomycin) and Ng et al. (2001) for *tetL* and *tetM* (tetracycline) (Chapter 6.2.9).

### 7.2.3 Tn916-Tn1545 PCR

Presence of the Tn916-Tn1545 was investigated to determine if this was a potential mechanism for gene transfer from the donor strains. The *int* gene in the transposon integrase gene family Tn916-Tn1545 was sought using the PCR method described in Gevers et al. (2003). The *E. faecalis* strains assessed were LMG 20790, LMG 20791, JH2-2, 1991b and 2004b. Positive controls were the two LMG cultures, and JH2-2 was the negative control. The primers were 5’-GCGTGATTGTATCTCACT-3’ (forward) and 5’-GACGCTCCTGTTGCTTCT-3’ (reverse), which produced a product size of 1028 bp. The DNA was isolated following the procedure in Chapter 6.2.2. The PCR used 30 cycles of denaturation (95 °C), annealing (50 °C) and extension (72 °C) for 1 min each. The polymerase enzyme (Chapter 6.2.3.1), equipment and staining (Chapter 6.2.4) were carried out as indicated in Chapter 6. PCR products were run on a 1 % agarose gel (Ultrapure Agarose, Life Technologies) at 100 V for 60 min.
Table 7.3 Carbohydrate fermentation properties of *Enterococcus* strains used to distinguish transconjugants in mating experiments

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Arabinose</th>
<th>Mannitol</th>
<th>α-cyclodextrin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Donors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> 1991b</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>E. faecalis</em> 2004b</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>E. faecalis</em> LMG 20790</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Recipients</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> JH2-2</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>E. faecium</em> 64/3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>E. hirae</em> 1981g</td>
<td>-</td>
<td>-</td>
<td>unknown</td>
</tr>
</tbody>
</table>
7.2.4 Biofilm mating

7.2.4.1 Growth of isolates in milk at 4, 10 and 25 °C

Prior to conducting transfer of antibiotic resistance genes in enterococci on a milk biofilm, the strains that were used for this experiment, *E. faecalis* 1991b, *E. faecalis* JH2-2, *E. faecium* 64/3 and *L. monocytogenes* E17, were assessed for their growth at the temperatures of 4, 10 and 25 °C. The strains were inoculated from slopes (enterococci on BHIA, *Listeria* on Tryptone Soya Yeast Extract Agar; Oxoid) into 10 mL BHIB and grown at 37 °C for 20 h. Cultures were diluted to ~$10^4$ cfu/mL using 0.1 % Bacteriological Peptone and then diluted a further 1 in 100 in full cream UHT milk to achieve a count of ~$10^2$ cfu/mL for each culture. The inoculated milk was dispensed in 10 mL aliquots into McCartney bottles and incubated at 4, 10 or 25 °C. Viable counts were performed weekly (4 °C), tri-weekly (10 °C) or daily (25 °C) by pour, spread (0.1 mL) or spiral plating serial dilution of cultures in 0.1 % Bacteriological Peptone, as described in Chapter 3.2.2. For 4 °C cultures, spread plates were also performed using 0.3 mL inocula. BHIA was incubated at 37 °C for 20-24 h and then colonies were counted.

7.2.4.2 Biofilm mating procedure

Biofilm mating was conducted on stainless steel coupons immersed in milk at 10 and 25 °C. The donor isolate was *E. faecalis* 1991b. Recipients were *E. faecalis* JH2-2, *E. faecium* 64/3 and *L. monocytogenes* E17. The recipients were established on the coupon initially, with the donor strain added to the milk surrounding the coupon. Recipient isolates were grown individually, from slopes, in BHIB at 37 °C for 20 h. Cultures were diluted 1 in 100 and added collectively to 60 mL of milk. The milk was poured onto four coupons sitting in a large Petri dish (14.2 cm diameter). The outside of the Petri dish was taped to prevent evaporation and spillage. It was incubated aerobically, without shaking, at 37 °C for 24 h ± 15 min. Coupons were removed and rinsed by gently pouring 100 mL of sterile water over the coupons. The coupons were placed singly inside 60 mL polystyrene containers (4.1 cm diameter).
The donor isolate was concurrently grown, from a slope, in BHIB supplemented with 10 µg/mL chloramphenicol, 4 µg/mL erythromycin and 10 µg/mL tetracycline for 22 h at 37 °C. It was then diluted to ~3.0 x 10^2 cfu/mL in full cream UHT milk and 15 mL was added into the polystyrene container with the coupon. The recipients and donor were incubated aerobically, without shaking, in experiments conducted at 10 and 25 °C. Mating at 10 °C was incubated for 21 d. Mating at 25 °C was incubated for 68 h. The experiment was replicated twice.

7.2.4.3 Recovery of transconjugants

After mating, the coupons were removed from the milk and rinsed with 100 mL of sterile deionised water. Cells were harvested with a swab that was moistened with 0.1 % Bacteriological Peptone. The swab was rubbed back and forth five times in one direction and then five times in the perpendicular direction. The swab was added to 5 mL of 0.1 % Bacteriological Peptone and vortexed. Both the swab solution and the incubated milk around the coupon were tested for transconjugants by serial diluting with 0.1 % Bacteriological Peptone and spread plating (0.1 mL) onto selective media containing antibiotics as well as media without the addition of antibiotics.

The medium used for the detection of enterococci was SBA, which can differentiate between *E. faecalis* and *E. faecium* (Figure 7.1). Each of the antibiotics was added individually to SBA at the levels used in the broth (Chapter 7.2.3.2). Fusidic acid and rifampin were also added to SBA as a mating control plate. *Listeria* do not grow on SBA. The medium used for the detection of *Listeria* was Oxford Agar. Each of the antibiotics was also added individually to Oxford Agar. Oxford Agar was the control plate for *Listeria*. Enterococci do not grow on Oxford Agar. All plates were incubated at 37 °C for 48 h.

7.2.5 Calculation of transfer frequency

The transfer frequency was the number of transconjugants on selective media divided by the number of recipients on control media. The control media were BHIA plates.
containing fusidic acid and rifampin (*E. faecalis* JH2-2 and *E. faecium* 64/3), BHIA containing nitrofurantoin and rifampin (*E. hirae* 1981grf3) and Oxford Agar (*Listeria*).

**7.3 Results**

**7.3.1 Tn916-Tn1545**

The transposon Tn916-Tn1545 was detected by PCR in one of the BCCM control cultures, LMG 20790 (Figure 7.2). It was absent from the negative control JH2-2. Of the two Australian raw milk *E. faecalis* isolates used for mating procedures, this transposon was detected in 1991b but not 2004b.

**7.3.2 MIC of antibiotics used in mating procedures**

In all cases, there was a multi-fold difference in the MIC values between isolates susceptible to an antibiotic and those resistant to an antibiotic (Table 7.4). The Australian raw milk isolates showed differences in the level of resistance to tetracycline. The isolate with the higher resistance, *E. faecalis* 1991b, had both the tetL and tetM genes whereas the isolate with slightly lower resistance, *E. faecalis* 2004b, only amplified the tetM gene. *E. faecalis* LMG 20790, a strain originally isolated from Cheddar cheese in Ireland (Huys et al., 2004), also only had the tetM gene. The *E. hirae* parent isolate, 1981g, was susceptible to all of these antibiotics, except nitrofurantoin, prior to selection for spontaneous mutation to rifampin resistance at ≥ 50 µg/mL.

**7.3.3 Filter mating**

**7.3.3.1 Chloramphenicol**

Chloramphenicol resistance was transferred to both the *E. faecalis* and the *E. faecium* recipients, but not to *E. hirae* or *L. monocytogenes* (Table 7.5). The expected carbohydrate fermentation properties of the presumptive transconjugants replica plated onto Phenol Red agar plates with α-cyclodextrin or arabinose (Table 7.3) confirmed that
Figure 7.1 *E. faecalis* JH2-2 (red) and *E. faecium* 64/3 (pink) differentiated on SBA

Figure 7.2 PCR of the transposon Tn916-Tn1545 in *E. faecalis*:

Lane 1) 100 bp ladder; 2) *E. faecalis* LMG 20790; 3) *E. faecalis* LMG 20791;
4) *E. faecalis* JH2-2; 5) *E. faecalis* 1991b; 6) *E. faecalis* 2004b; 7) water

Table 7.4 MIC of enterococci on BHIA for antibiotics used in mating procedures

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Antibiotic (µg/mL)</th>
<th>Donors</th>
<th>Ery</th>
<th>Fus</th>
<th>Rif</th>
<th>Strep</th>
<th>Tet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> 1991b</td>
<td>≥ 16</td>
<td>1</td>
<td>≥ 16</td>
<td>≤ 16</td>
<td>16</td>
<td>≥ 2000</td>
<td>≥ 64</td>
</tr>
<tr>
<td><em>E. faecalis</em> 2004b</td>
<td>≥ 16</td>
<td>1</td>
<td>≥ 16</td>
<td>≤ 16</td>
<td>16</td>
<td>≥ 2000</td>
<td>50</td>
</tr>
<tr>
<td><em>E. faecalis</em> LMG 20790</td>
<td>≥ 16</td>
<td>1</td>
<td>≥ 16</td>
<td>≤ 16</td>
<td>16</td>
<td>≤ 125</td>
<td>≥ 64</td>
</tr>
<tr>
<td>Recipients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> JH2-2</td>
<td>≤ 4</td>
<td>2</td>
<td>≥ 128</td>
<td>≥ 256</td>
<td>≤ 125</td>
<td>≤ 16</td>
<td></td>
</tr>
<tr>
<td><em>E. faecium</em> 64/3</td>
<td>≤ 4</td>
<td>2</td>
<td>≥ 128</td>
<td>≥ 256</td>
<td>≤ 125</td>
<td>≤ 16</td>
<td></td>
</tr>
<tr>
<td><em>E. hirae</em> 1981g parent</td>
<td>≤ 4</td>
<td>2</td>
<td>≤ 16</td>
<td>≤ 16</td>
<td>≤ 125</td>
<td>≤ 16</td>
<td></td>
</tr>
</tbody>
</table>

1 Ery = erythromycin, Fus = fusidic acid, Rif = rifampin, Strep = streptomycin, Tet = tetracycline; Chloramphenicol concentration used as determined in Chapter 6.3.4 and as used in Huys et al. (2004)
the strains on the antibiotic selective media were not the donor strains. Furthermore, the multiple phenotypic resistance (Table 7.5) indicates that the strains were presumptive transconjugants as it would be unlikely that the recipient strains would spontaneously acquire multiple resistances to three antibiotics. The frequency of transconjugation was calculated as chloramphenicol-resistant colonies per recipient. Selected colonies were then genetically screened by PCR for the cat gene and phenotypically screened for other resistances transferred. PCR confirmation of genes showed required reactions for the positive (donor) and negative (recipient) controls, as seen in the cat PCR previously (Chapter 6.3.4.4, Figure 6.7). Erythromycin and tetracycline resistance were transferred concurrently to both recipients. Streptomycin resistance was also transferred to 100 % of the E. faecium transconjugants but to only 22 % of the E. faecalis transconjugants. The cat gene was detected in 100 % of the E. faecium transconjugants but in none of the E. faecalis transconjugants.

7.3.3.2 Erythromycin

All three donors transferred the ermB gene to each of the enterococci recipients (Table 7.6), which were confirmed as presumptive transconjugants from their carbohydrate fermentation reactions on Phenol Red agar with α-cyclodextrin, arabinose or mannitol (Table 7.3). The highest transfer frequency was from E. faecalis LMG 20790 to E. faecium 64/3. The erythromycin resistance gene, ermB, was most consistently transferred to the E. hirae and E. faecium recipients, with a lesser detection of the gene in E. faecalis. Transfer of multiple phenotypic resistance occurred from donor E. faecalis 1991b, which transferred all of the other three resistances (Table 7.6). Although the E. hirae recipient had ermB in 100 % of the transconjugants, none of these acquired resistance to the other antibiotics. E. faecalis 2004b, which additionally had tetracycline and streptomycin resistance, did not consistently transfer multiple resistance. The E. faecalis LMG 20790 donor had an additional tetracycline resistance, but this was not co-transferred.
Table 7.5 Transfer of chloramphenicol resistance (*cat*) from the *E. faecalis* 1991b raw milk strain on filters

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Transfer frequency (±SD)</th>
<th><em>cat</em> gene detection (%)</th>
<th>Multiple phenotypic resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. faecalis</em> 1991b³</td>
<td><em>E. faecalis</em></td>
<td>9.7 (± 6.4) x 10⁻⁵</td>
<td>0</td>
<td>100 % Ery, 22 % Strep, 99 % Tet</td>
</tr>
<tr>
<td></td>
<td><em>E. faecium</em></td>
<td>3.4 (± 4.8) x 10⁻⁵</td>
<td>100</td>
<td>100 % Ery, Strep, Tet</td>
</tr>
<tr>
<td></td>
<td><em>E. hirae</em></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>L. monocytogenes</em></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

¹ SD = Standard deviation

² Ery = erythromycin, Strep = streptomycin, Tet = tetracycline

³ 1991b has chloramphenicol, erythromycin, streptomycin and tetracycline resistance

Table 7.6 Transfer of erythromycin resistance (*ermB*) from *E. faecalis* 1991b, *E. faecalis* 2004b and *E. faecalis* LMG 20790 on filters

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Transfer frequency (±SD)</th>
<th><em>ermB</em> gene detection (%)</th>
<th>Multiple phenotypic resistance²</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. faecalis</em> 1991b³</td>
<td><em>E. faecalis</em></td>
<td>2.7 (± 0.2) x 10⁻⁴</td>
<td>45</td>
<td>100 % Chlor, 11 % Strep, 99 % Tet</td>
</tr>
<tr>
<td></td>
<td><em>E. faecium</em></td>
<td>3.5 (± 3.2) x 10⁻⁸</td>
<td>90</td>
<td>100 % Chlor, Strep, Tet</td>
</tr>
<tr>
<td></td>
<td><em>E. hirae</em></td>
<td>2.4 (± 0.9) x 10⁻⁶</td>
<td>100</td>
<td>0 % Chlor, Strep, Tet</td>
</tr>
<tr>
<td></td>
<td><em>L. monocytogenes</em></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> 2004b⁴</td>
<td><em>E. faecalis</em></td>
<td>7.7 (± 1.2) x 10⁻⁵</td>
<td>60</td>
<td>0 % Tet, no strep results⁵</td>
</tr>
<tr>
<td></td>
<td><em>E. faecium</em></td>
<td>6.7 (± 1.6) x 10⁻⁵</td>
<td>100</td>
<td>100 % Strep, 0% Tet</td>
</tr>
<tr>
<td></td>
<td><em>E. hirae</em></td>
<td>1.3 (± 0.2) x 10⁻⁶</td>
<td>100</td>
<td>0 % Strep, Tet</td>
</tr>
<tr>
<td><em>E. faecalis</em> LMG 20790⁶</td>
<td><em>E. faecalis</em></td>
<td>6.6 (± 4.8) x 10⁻⁵</td>
<td>60</td>
<td>0 % Tet</td>
</tr>
<tr>
<td></td>
<td><em>E. faecium</em></td>
<td>1.2 (± 0.7) x 10⁻⁶</td>
<td>100</td>
<td>0 % Tet</td>
</tr>
<tr>
<td></td>
<td><em>E. hirae</em></td>
<td>2.9 (± 0.4) x 10⁻⁶</td>
<td>100</td>
<td>0 % Tet</td>
</tr>
</tbody>
</table>

¹ SD = Standard deviation

² Chlor = chloramphenicol, Strep = streptomycin, Tet = tetracycline

³ 1991b has chloramphenicol, erythromycin, streptomycin and tetracycline resistance

⁴ 2004b has erythromycin, streptomycin and tetracycline resistance

⁵ Streptomycin plate was unreadable due to smearing

⁶ LMG 20790 has erythromycin and tetracycline resistance

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7.3.3.3 Streptomycin

Transfer of streptomycin resistance occurred to *E. faecalis* and *E. faecium*, but not to *E. hirae* from both donors (Table 7.7). Recipients were confirmed as presumptive transconjugants from their α-cyclodextrin or arabinose fermentations on Phenol Red agar (Table 7.3). However, transconjugants from the isolate *E. faecalis* 1991b with a known streptomycin resistance gene (*ant*(6)-I) did not amplify the gene which may indicate that a currently undefined mobile resistance gene was transferred. The cause of streptomycin resistance in *E. faecalis* 2004b was unknown. The highest transfer frequency was from this isolate to *E. faecium* 64/3. Multiple resistance was transferred to both *E. faecalis* and *E. faecium* from *E. faecalis* 2004b but only to *E. faecalis* from *E. faecalis* 1991b.

7.3.3.4 Tetracycline

Tetracycline resistance was transferred to all recipients, although at different frequencies (Table 7.8). Presumptive enterococci transconjugants were confirmed from their carbohydrate fermentation reactions on Phenol Red agar with α-cyclodextrin, arabinose or mannitol (Table 7.3). The presumptive *L. monocytogenes* transconjugants was obtained from Oxford agar. Transfer to *E. hirae* was infrequent, with no transfers from *E. faecalis* 1991b, and transfer in only one of the two matings with both *E. faecalis* 2004b and *E. faecalis* LMG 20790. The *tetL* gene was transferred solely from *E. faecalis* 1991b to the *E. faecalis* recipient, along with multiple resistances. This was the only incidence of *tetL* transfer. It was also the highest transfer frequency. All of the other gene transfers were of *tetM*, including to *L. monocytogenes* E17. This was the only resistance that was transferred to *L. monocytogenes* E17. The *tetM* gene was predominantly transferred without multiple resistances. In all instances where genes were transferred, the genes were detected in 100% of the transconjugants.
Table 7.7 Transfer of streptomycin resistance from *E. faecalis* 1991b (*ant*(6)-I) and *E. faecalis* 2004b on filters

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Transfer frequency (±SD)(^1)</th>
<th><em>ant</em>(6)-I gene detection (%)</th>
<th>Multiple phenotypic resistance(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. faecalis</em> 1991b(^3)</td>
<td><em>E. faecalis</em></td>
<td>2.6 (± 2.2) × 10(^{-6})</td>
<td>0</td>
<td>100 % Chlor, Ery, 99 % Tet</td>
</tr>
<tr>
<td></td>
<td><em>E. faecium</em></td>
<td>4.5 (± 6.4) × 10(^{-9})</td>
<td>0</td>
<td>0 % Chlor, Ery, Tet</td>
</tr>
<tr>
<td></td>
<td><em>E. hirae</em></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>L. monocytogenes</em></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> 2004b(^4)</td>
<td><em>E. faecalis</em></td>
<td>9.6 (± 2.0) × 10(^{-6})</td>
<td>unknown</td>
<td>100 % Ery, 0 % Tet</td>
</tr>
<tr>
<td></td>
<td><em>E. faecium</em></td>
<td>3.4 (± 1.2) × 10(^{-5})</td>
<td>unknown</td>
<td>99 % Ery, 0 % Tet</td>
</tr>
<tr>
<td></td>
<td><em>E. hirae</em></td>
<td>0</td>
<td>unknown</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) SD = Standard deviation

\(^2\) Chlor = chloramphenicol, Ery = erythromycin, Tet = tetracycline

\(^3\) 1991b has chloramphenicol, erythromycin, streptomycin and tetracycline resistance

\(^4\) 2004b has erythromycin, streptomycin and tetracycline resistance
Table 7.8 Transfer of tetracycline resistance from *E. faecalis* 1991b (*tetL, tetM*), *E. faecalis* 2004b (*tetM*) and *E. faecalis* LMG 20790 (*tetM*) on filters

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Transfer frequency (±SD)</th>
<th><em>tetL or tetM</em> gene detection (%)</th>
<th>Multiple phenotypic resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(±SD)¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> 1991b²</td>
<td><em>E. faecalis</em></td>
<td>2.2 (± 2.0) x 10⁻⁴</td>
<td>100 (<em>tetL</em>)</td>
<td>99 % Chlor, 100 % Ery, 63 % Strep</td>
</tr>
<tr>
<td></td>
<td><em>E. faecium</em></td>
<td>3.0 (± 0.7) x 10⁻⁷</td>
<td>0 (*tetL)</td>
<td>2 % Chlor, 0 % Ery, 0 % Strep</td>
</tr>
<tr>
<td></td>
<td><em>E. hirae</em></td>
<td>0</td>
<td>100 (<em>tetM</em>)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>L. monocytogenes</em></td>
<td>1.1 (± 0.1) x 10⁻⁷</td>
<td>0 (*tetL)</td>
<td>0 % Chlor, 0 % Ery, 0 % Strep</td>
</tr>
<tr>
<td></td>
<td><em>E. faecalis</em> 2004b³</td>
<td>8.3 (± 1.6) x 10⁻⁸</td>
<td>0 (*tetL)</td>
<td>0 % Ery, no Strep results⁴</td>
</tr>
<tr>
<td></td>
<td><em>E. faecium</em></td>
<td>0</td>
<td>100 (<em>tetM</em>)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>E. hirae</em></td>
<td>2.5 x 10⁻⁷*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>E. faecalis</em> LMG 20790⁵</td>
<td>3.3 (± 3.9) x 10⁻⁶</td>
<td>100 (<em>tetM</em>)</td>
<td>0 % ery</td>
</tr>
<tr>
<td></td>
<td><em>E. faecium</em></td>
<td>2.9 (± 0.1) x 10⁻⁷</td>
<td>100 (<em>tetM</em>)</td>
<td>4 % ery</td>
</tr>
<tr>
<td></td>
<td><em>E. hirae</em></td>
<td>4.7 x 10⁻⁷*</td>
<td>100 (<em>tetM</em>)</td>
<td>0 % ery</td>
</tr>
</tbody>
</table>

¹ SD = Standard deviation

² Chlor = chloramphenicol, Ery = erythromycin, Strep = streptomycin

³ 1991b has chloramphenicol, erythromycin, streptomycin and tetracycline resistance

⁴ Streptomycin plate was unreadable due to smearing

⁵ 2004b has erythromycin, streptomycin and tetracycline resistance

* Transfer occurred only in 1 of the 2 experiments

** Presumptive transconjugant obtained did not survive for further testing
7.3.4 Growth of isolates in milk at 4, 10 and 25 °C

At the coldest temperature assessed, 4 °C, the *Listeria* isolate grew more quickly than all of the enterococci isolates (Figure 7.3). Of the enterococci isolates, only the *E. faecium* isolate was able to grow. Neither of the *E. faecalis* isolates increased in number after five weeks of incubation and appeared to be losing viability. All of the isolates were able to grow at both 10 °C and 25 °C (Figure 7.3). The two dairy isolates, *E. faecalis* 1991b and *L. monocytogenes* E17, grew the fastest in milk at 10 °C. By 21 days at 10 °C, all of the isolates reached stationary phase. Similarly, all of the isolates reached stationary phase at or before two days at 25 °C, at which point they had a comparable total count.

7.3.5 Biofilm mating experiments

Since both *E. faecalis* isolates used in the biofilm mating did not grow at 4 °C (Figure 7.3), biofilm mating was only conducted at 10 and 25 °C. The donor (*E. faecalis* 1991b) and three recipient strains (*E. faecalis* JH2-2, *E. faecium* 64/3 and *L. monocytogenes* E17) were recovered from both the coupon and the surrounding milk in the biofilm transfer experiment, however no transconjugants were detected either in the milk or in the biofilm in which the recipients were established on the coupon. Recovery of total enterococci was \(10^{5}-10^{6}\) cfu/mL of swab solution from the coupon and \(10^{8}\) cfu/mL in the surrounding milk at 10 °C (Figure 7.4). The 25 °C mating yielded \(10^{4}-10^{5}\) cfu/mL enterococci in the swab solution from the coupon and \(10^{8}\) cfu/mL in the surrounding milk. A greater number of enterococci were recovered from the coupon biofilm at 10 °C compared to 25 °C despite similar counts in the milk at both temperatures.

*L. monocytogenes* counts were consistently at \(10^{7}\) cfu/mL in the milk at both temperatures; however, recovery from the biofilm on the coupons was different at the two temperatures. Although *L. monocytogenes* had the fastest growth at the lower temperature of 10 °C (Figure 7.3), it did not produce a detectable biofilm at this temperature. *L. monocytogenes* counts on the coupon were below the limit of detection (1 log_{10} cfu/ml) in both replicates at 10 °C (Figure 7.4). At 25 °C, *L. monocytogenes* counts on the coupon were between \(10^{2}-10^{3}\) cfu/mL.
Figure 7.3 Growth of *E. faecalis* 1991b, *E. faecalis* JH2-2, *E. faecium* 64/3 and *L. monocytogenes* E17 in milk at: (a) 4 °C (b) 10 °C (c) 25 °C
Figure 7.4 Growth of *E. faecalis* (1991b and JH2-2), *E. faecium* (64/3) and *L. monocytogenes* (E17) during biofilm mating in UHT milk and on a stainless steel coupon after incubation at: (a) 10 °C for 21 d (b) 25 °C for 68 h

Error bars are the standard deviation of the replicate experiments and are not visible when the standard deviation was low.
7.4 Discussion

7.4.1 Filter mating at optimal temperature

Chloramphenicol acetyltransferase, streptomycin adenyltransferase and tetracycline and erythromycin resistance genes have been reported to occur concurrently on plasmids in enterococci (Courvalin et al., 1978). All four of these resistances were found in *E. faecalis* 1991b. When the primary selected resistance in the filter mating experiments was either chloramphenicol or erythromycin, phenotypic resistance to these along with tetracycline was present in both the *E. faecalis* and *E. faecium* transconjugants. This suggests that these genes may be located together in *E. faecalis* 1991b and could be co-transferring. Unlike the *E. faecium* and *E. hirae* transconjugants, the *cat* and *ermB* genes were not always detected in the *E. faecalis* transconjugants from mating experiments with chloramphenicol and erythromycin selection. The presumptive transconjugants exhibited carbohydrate fermentation reactions consistent with the recipients, and it is possible that they had developed spontaneous mutation to the antibiotics. The insertion sequence IS256 has lead to the development of erythromycin and chloramphenicol resistance in *S. aureus*, and this insertion sequence has also been found in *E. faecalis* (Schreiber et al., 2013).

On the other hand, when the primary selected resistance was tetracycline, co-transfer of erythromycin and chloramphenicol resistance only occurred with the *E. faecalis* JH2-2 recipient. In this case, the resistance determinant was *tetL*, not *tetM*. All of the *tetM* transconjugants produced from all three *E. faecalis* donor isolates had negligible co-transfer of other antibiotic resistances, which is contrary to the resistances being linked on the donor isolate. The *tetL* gene is for an efflux protein located on transferable plasmids while *tetM* is a ribosomal protection protein that can be located on the chromosome or plasmids (Teuber et al., 1999; Chopra and Roberts, 2001). This then suggests that in *E. faecalis* 1991b, genes for chloramphenicol (*cat*) and erythromycin (*ermB*) resistance may be located on a transferable plasmid with *tetL*. The donor *E. faecalis* LMG 20790 has *ermB* and *tetM* and also has a transposon in the Tn916-
Tn1545 family (Huys et al., 2004). In the filter mating experiments of Huys et al. (2004), \textit{ermB}, \textit{tetM} and Tn916-Tn1545 were transferred from \textit{E. faecalis} LMG 20790 to \textit{E. faecalis} JH2-2. In the current experiments, the erythromycin and tetracycline resistance were generally not co-transferred between these isolates.

In the transfer of tetracycline resistance to \textit{E. faecalis} from enterococci obtained from food, it has been reported that \textit{tetM} transferred more frequently than \textit{tetL} (Huys et al., 2004), and conversely, that \textit{tetL} transferred more frequently than \textit{tetM} (Wilcks et al., 2005). These studies used one recipient each. The current study had fewer donor isolates but multiple recipient isolates and found that \textit{tetM} transferred more frequently than \textit{tetL} and was influenced by the recipients, as seen in antibiotic resistance transfer in other genera (Van Meervenne et al., 2012).

Streptomycin resistance was transferred to several recipients; however, the \textit{ant}(6)-I gene was not detected in the \textit{E. faecalis} 1991b transconjugants which may indicate that a currently undefined mobile resistance gene was transferred. From this donor, transconjugants formed with the \textit{E. faecalis} JH2-2 recipient were usually multiply resistant to the other antibiotics. On the other hand, \textit{E. faecium} 64/3 transconjugants were only multiply resistant when the primary selection was with chloramphenicol or erythromycin, but not when the primary selection was streptomycin or tetracycline. Similarly, the \textit{E. faecalis} 2004b donor, which did not have chloramphenicol resistance, only co-transferred streptomycin and erythromycin, indicating that there could be a relationship between the genes for these resistances in this donor isolate. In clinical \textit{E. faecium} isolates, gentamicin, vancomycin, erythromycin and streptomycin resistance were co-transferred to \textit{E. faecium} recipients along with a hyaluronidase virulence factor, where erythromycin and streptomycin transferred separately to the plasmid containing the virulence factor and other antibiotic resistances (Arias et al., 2009). It was not determined whether the erythromycin and streptomycin genes (\textit{ermB} and \textit{ant}(6)-I) were linked, but it was suspected that they were on the same plasmid (Arias et al., 2009). Another mechanism for streptomycin resistance has been identified in \textit{S. aureus}, where streptomycin selected for mutations in genes associated with cell wall synthesis and
maintenance (Johnston et al., 2016), and may be a reason for the unknown resistance mechanism in the current work.

\textit{E. faecalis} and \textit{E. faecium} are more often the enterococci species investigated in regards to antibiotic resistance transfer. With the possibility of \textit{E. hirae} being pathogenic (Bourafa et al., 2015; Dicpinigaitis et al., 2015), transfer of antibiotic resistance genes to this species is also a concern. This species is infrequently assessed in antibiotic resistance transfer determinations. \textit{E. hirae} isolated from meat has been used as a donor and was able to transfer erythromycin resistance (\textit{ermAM}) at a lower frequency ($10^{-7}$) to \textit{E. faecalis} JH2-2 (Teuber et al., 1999). An \textit{E. hirae} isolate with multiple tetracycline resistance determinants (\textit{tetK, tetL, tetM} and \textit{tetO}) from pig faeces was not shown to transfer resistance to \textit{E. faecalis} OG1RF (Rizzotti et al., 2009). The current work is, to my knowledge, the first instance of successful antibiotic resistance transfer to \textit{E. hirae}, which is significant in context of this species increased resistance to heat treatment (Chapter 3.3.2.4). Both erythromycin and tetracycline resistance were transferred with 100% of the \textit{E. hirae} transconjugants possessing the \textit{ermB} or \textit{tetM} genes, showing efficient transfer of the genes when transfer occurred.

\textbf{7.4.2 Growth at environmental temperatures}

The temperatures considered for biofilm mating were chosen as they related to environmental temperatures that are feasibly encountered in the dairy industry. The guidelines for food safety for dairy manufacturers recommends that dairy foods be kept at or below 5 °C (Dairy Food Safety Victoria, 2006) and, as such, 4 °C is a typically used temperature for storage. As an abuse of this guideline, 10 °C was chosen to represent poor storage and is also the temperature which was one of the original defining characteristics of the genus (Devriese and Pot, 1995). Then 25 °C was used to represent ambient temperature outside of dairy equipment. The optimum growth temperature of 37 °C (Giraffa, 2014) was not used as it is unlikely that milk will reach this temperature normally given mean temperatures in Victoria (Appendix E).
The two *E. faecalis* isolates were not able to grow at 4 °C. This is consistent with the 4 and 7 °C screening experiments where the majority of *E. faecalis* had weak growth at 4 °C (Chapter 4.3.1.1). Although some *E. faecalis* would be able to grow at this temperature (Chapter 4.3.3, Figure 4.3), this was not the case for *E. faecalis* 1991b. As the notional minimum temperature of *E. faecalis* JH2-2 has been estimated to be 3.6 °C (Thammavongs et al., 1996), growth at 4 ± 1 °C would be a challenge and not expected. All of the isolates grew at 10 and 25 °C, so the milk biofilm experiments proceeded at these temperatures.

### 7.4.3 Biofilms and enterocins

A further consideration with the mixed species biofilm is the potential for enterococci to produce bacteriocins, called enterocins (Khan et al., 2010), and what effect that might have on the *L. monocytogenes* in the biofilm. Enterocins have activity against *L. monocytogenes* (Khan et al., 2010). It has been reported that enterococci in a biofilm with *L. monocytogenes* on stainless steel inhibited *L. monocytogenes* (Zhao et al., 2004, 2013). In addition, *L. monocytogenes* was reduced or eliminated in poultry processing factory floor drains that were treated with *Lactococcus lactis* and *E. durans*, which had been shown previously to have anti-listerial activity (Zhao et al., 2013). However, other studies found that *L. monocytogenes* was able to grow to higher levels when present in an enterococci biofilm possibly due to the exopolysaccharide products of the enterococci that aided the adhesion of *L. monocytogenes* to the surface (Fernandes et al., 2015). Similarly, the presence of *E. gallinarum* in poultry factory drains increased the growth and colonisation of *Listeria* in the drains (Fox et al., 2014). Therefore the effect of enterococci on *Listeria* in biofilms is strain dependent, where the presence of enterococci can aid *Listeria* growth in biofilms in some instances, yet inhibit *Listeria* in other instances where enterocins are present. The assessment of bacteriocins in enterococci was outside the scope of the present study but it would be of interest to know what the ability of the dairy enterococci strains were in regards to the inhibition of other bacteria encountered in dairy factories.
7.4.4 Attempted biofilm mating at environmental temperatures

*E. faecalis* formed more biofilm at 10 °C than at 25 °C and was better at forming biofilms than *L. monocytogenes* at both temperatures. At 10 °C, *L. monocytogenes* was not isolated from a biofilm as numbers were below the limit of detection (1 log$_{10}$ cfu/mL). It has been shown with a variety of *L. monocytogenes* that biofilm production is greater at higher temperatures (Nilsson et al., 2011; Kadam et al., 2013). Another study failed to isolate *E. faecalis, E. faecium* or *L. monocytogenes* from biofilms on stainless steel coupons in a dairy medium at 7 °C but obtained significantly more biofilm growth at 25 °C (Fernandes et al., 2015). The difference in temperature between that study at 7 °C and the current study at 10 °C is possibly an influencing factor since 7 °C will be near the lower limit for enterococci growth (Giraffa, 2014). On the other hand, studies of food enterococci biofilms in Tryptone Soya Broth on polystyrene have had mixed outcomes, where enterococci biofilms increased with increasing temperature (10, 28 and 37 °C) in one study (Marinho et al., 2013) whereas the enterococci formed more biofilm at 25 °C than at 37 °C in other work (Jahan and Holley, 2014). While additional nutrients in the form of glucose may assist enterococci in forming stronger biofilms (Marinho et al., 2013), *L. monocytogenes* forms stronger biofilms in nutrient-poor media (Kadam et al., 2013). This phenomenon has also been seen with enterococci, where they formed stronger biofilms in nutrient-poor media (Kristich et al., 2004). The medium used in the current experiment, milk, would be nutrient-rich. So the conditions in the current system using a nutrient-rich medium at lower temperatures could favour enterococci biofilms if the enterococci were strains that performed better with additional nutrients.

Although enterococci mating occurred on filters at an optimum 37 °C, this was not the case in the milk biofilm at 10 and 25 °C, where no transconjugants were detected. Filter mating experiments at 10 and 25 °C would help determine if growth temperature is a factor for why transconjugants were not obtained in the biofilm mating experiment. Other work found that filter mating between *Lactobacillus* and *L. monocytogenes* was not inhibited at lower temperatures (7 and 10 °C) provided that sufficient time (5-10 d) was allowed for the mating to occur (Van Meervenne et al., 2015). Conversely, mating between *E. faecalis* and *L. innocua* was successful on meat slices at 30 °C but not at 10 °C.
showing that lower temperatures can inhibit bacterial mating between some strains. Few studies have conducted enterococci mating experiments in milk. Chloramphenicol resistance has been transferred on a conjugative plasmid from *E. faecalis* to *E. italicus* in milk at a similar transfer frequency to what was observed in filter mating (Borgo et al., 2009). Gene transfer can occur efficiently in biofilms, although recipients on the inside of a bacterial biofilm may not receive genetic material if they are not in contact with the donors on the outside of the biofilm (Molin and Tolker-Nielsen, 2003). An example of this was seen in the antibiotic resistance transfer between *E. faecalis* isolates where the transfer frequency was reduced in the biofilm phase compared to liquid culture using BHIB (Cook et al., 2011). These suggest that mating may not be inhibited by lower temperatures or in a milk medium, but could be influenced by interactions within a biofilm. There could, of course, be strain variations for any of these factors, as the enterococci in this study grew in milk at lower temperatures (10 °C) but did not produce detectable transconjugants either in milk or on a biofilm.

### 7.5 Conclusion

In the current study, the most frequently encountered antibiotic resistances present in *E. faecalis* isolated from Australian raw milk were transferred to *E. faecalis*, *E. faecium*, *E. hirae* and *L. monocytogenes* via filter mating at 37 °C. Erythromycin, streptomycin and tetracycline resistance could be transferred to both *E. faecalis* and *E. faecium* but was limited to *E. hirae* and *L. monocytogenes*. Chloramphenicol resistance was only transferred successfully to *E. faecium*. The transfer of specific tetracycline resistance genes was influenced by the recipient. Streptomycin resistance was acquired by *E. faecalis* and *E. faecium* but was due to an undefined mobile resistance gene, as the known gene, ant(6)-I, was not detected in the transconjugants. The transfer of multiple resistances was not consistent and indicates that the genes are not necessarily co-associated. Growth of the enterococci in milk was limited at 4 °C. Biofilm production of *E. faecalis* was greater at 10 °C than at 25 °C compared to the growth in milk. Although multiple antibiotic resistances transferred in laboratory filter mating, the transfer of
antibiotic resistance from enterococci on a milk biofilm was not detected under the experimental conditions used.
CHAPTER 8 - GENERAL DISCUSSION
8.1 Summary of main findings

This research was undertaken to determine the incidence of enterococci in raw milk and dairy products in the dairying regions of south-eastern Australia over a one-year period and to assess the ability of enterococci to persist in the dairy environment, survive pasteurisation and grow in dairy products. The risks associated with enterococci in dairy products due to the presence of virulence factors, antibiotic resistance and the ability to transfer antibiotic resistance was also examined.

There is no method enshrined in a standard for the detection of enterococci in food. Prior to the commencement of sampling milk from raw milk silos, commercial media were assessed for their ability to differentiate dairy enterococci from other related bacteria and for recovery of verified Enterococcus species on the media. A selection of dairy enterococci and similar microorganisms from the CSIRO culture collection was used for this purpose. Chromocult broth with agar provided good recovery of and was less inhibitory to the enterococci than the other media assessed. Enterococci formed blue colonies on this chromogenic medium; however, as for all of the media assessed, traditional physiological confirmation tests (Chapter 2) were required. Chromocult agar was subsequently used for the year-long survey of raw milk and dairy products (Chapter 2).

A survey of the incidence of antibiotic resistance was undertaken in 2002, concurrent to the year-long raw milk survey, using dairy enterococci from the CSIRO culture collection. The enterococci had been isolated from raw milk tankers using Enterococcosel agar as detailed in Chapter 8.2. This collection was later studied in terms of determining the presence of virulence factors and selected isolates were used to determine whether antibiotic resistances were transferable between enterococci and L. monocytogenes (Chapter 6). The most frequently encountered antibiotic resistances in this collection were to tetracycline followed by streptomycin, commonly in the same strains (Chapter 6.3.5). Vancomycin resistance was not detected in any of the enterococci other than the inherently resistant species E. gallinarum. Ampicillin resistance was infrequent, and penicillin resistance was not detected. The most frequently encountered resistances,
streptomycin and tetracycline, along with chloramphenicol and erythromycin resistance which were also present in some of the dairy strains, could be transferred from *E. faecalis* to *E. faecium* by filter mating but in limited situations to *E. faecalis*, *E. hirae* and *L. monocytogenes* (Chapter 7). *E. hirae* acquired erythromycin (*ermB*) and tetracycline (*tetM*) resistance but only tetracycline resistance (*tetM*) transferred to *L. monocytogenes*. Notably, laboratory antibiotic resistance transfer into *E. hirae* has not been reported previously. This is important given that *E. hirae* and other thermotolerant strains could survive pasteurisation and grow at refrigeration storage temperatures in pasteurised milk (Chapter 4.3.3). *E. faecalis*, *E. faecium* and *L. monocytogenes* were also mated in biofilms on stainless steel coupons in milk at 10 and 25 °C, but no transconjugants were detected under these experimental conditions used.

Virulence factors were predominantly found in *E. faecalis*, with β-haemolysis and gelatinase only detected in this species (Chapter 6). The *esp* and *agg* genes were found in the same number of isolates; however, strains were twice as likely to have *esp* than *agg* if only one of the four virulence factors was present. The presence of multiple virulence factors (three or more) more often occurred in strains that did not exhibit resistance to any of the medically-important antibiotics and were only detected in *E. faecalis*, which could survive pasteurisation treatments (Chapter 3.3.2.2). When antibiotic resistance did occur in strains with multiple virulence factors, the resistance was to tetracycline.

Concurrent to the determination of the incidence of antibiotic resistance in enterococci from raw milk tankers, a year-long survey was conducted of enterococci in raw milk and dairy products (Chapter 2). Raw milk was obtained at approximately monthly intervals from silos at six Victorian dairy factories, with pasteurised milk also obtained from one of these factories. Raw milk predominantly contained *E. faecalis*, with *E. faecium* being the second most common species, although *E. durans* had the highest incidence in pasteurised milk samples. Temporal analysis found that enterococci counts in raw milk were lower in winter, which has not been otherwise reported internationally. The enterococci counts were not different between the three regions (north, east, and west of Melbourne) but there were differences between individual factories, with some factories having higher counts. Enrichment of pasteurised milk allowed lower numbers to
be detected (1 cfu/100 mL). Although the prevalence of enterococci in pasteurised milk was low, thermoduric strains could survive the process and grow in milk at refrigerated temperatures (Chapter 4.3.3). Milk products were assessed quarterly and were shown to contain lower numbers and fewer species of enterococci than raw milk and required enrichment using 10 or more grams of butter, Cheddar cheese and milk powder in order to detect the enterococci. Cheddar cheese showed the widest variety of enterococci species, with four species detected, whereas *E. faecium* was the only species detected in the milk powder. Despite the predominance of *E. faecalis* in the raw milk, the species with the highest incidences in the greatest number in butter, Cheddar cheese and milk powder samples were *E. faecalis, E. malodoratus* and *E. faecium*, respectively.

Thermotolerance ranking was performed on one isolate of each of the four main species, *E. faecalis, E. faecium, E. hirae* and *E. durans*, identified from laboratory-pasteurised milk (63 °C/30 min) from each sampling time at each factory (Chapter 3). Thermal death determinations of strains with mid- and high-ranking thermotolerance for each species were conducted in an immersed coil apparatus. *E. faecalis* strains were most heat sensitive, with z values ranging from 5.0 to 7.5 °C. The highest z values, and therefore heat resistances, were observed in *E. durans* (8.7 and 8.8 °C), *E. faecium* (9.0 °C) and *E. hirae* (8.5 and 9.8 °C). This explains why *E. faecium* was detected in the milk powder despite the majority of enterococci in raw milk being *E. faecalis* (74.3 %) (Chapter 2). *E. faecium* and *E. hirae* had a similar incidence in raw milk, 8.6 and 8.5 %, respectively but it was unknown why *E. hirae* was not detected in the milk powder, given their similar heat resistances. It is possible that *E. faecium* is more resistant to desiccation that *E. hirae*.

The thermoduric enterococci characterised in Chapter 3 were grown in milk at 4 and 7 °C (Chapter 4). All of the strains were able to grow at both temperatures, although *E. faecalis* showed weaker growth at 4 °C compared to the other species. In butter, Cheddar cheese and milk powder that were naturally contaminated with enterococci, the majority of samples had decreased enterococci levels at the end of product storage (six, six and 12 months, respectively). When the thermoduric enterococci were inoculated into yoghurt and cottage cheese, they were able to survive but not grow at 4 and 7 °C, but decreased in number in yoghurt at both temperatures after three weeks. This may have been due
to the low pH of the yoghurt as pH below pH 5.5 was bacteriocidal to the enterococci at 4 °C (Chapter 4.3.1.2.3).

Genetic fingerprinting of enterococci from the raw milk silos at one of the dairy factories over a nine-month period identified a persistent population of *E. faecalis*, which had streptomycin resistance and increased biofilm production, and inferred a link between these traits and persistence in the raw milk silos at the factory assessed (Chapter 5). Erythromycin and tetracycline resistance were more commonly associated with the formation of moderate or weak biofilms by enterococci. Enterococci that formed weak or non-adherent biofilms could also be persistent, which could be due to milk residues in difficult to clean niches in the equipment that re-introduced enterococci into subsequent batches of milk.

Although the survey of raw milk and dairy products was conducted in 2002-2003, the results reported in this study are still of interest as this is the only study of this kind in Australia, to my knowledge. The persistence study reported in Chapter 5 showed that strains of enterococci persisted in the raw milk silos over time, and as practices in the Victorian dairy industry will not have changed significantly in this time, these results are still relevant today. In addition, as mentioned in Chapter 6.4.5.3, this study provides baseline data for changes that may occur due to changes in animal stock, antibiotic use and industry practices and could be used to monitor the emergence of antibiotic resistant enterococci in the Victorian dairy industry.

### 8.2 Collections of microorganisms

The collection of enterococci used for media assessment and antibiotic resistance and virulence factor determinations was obtained in 2001, not long after an external survey had been conducted on antibiotic usage in Victorian dairy cattle herds (Dairy Industry Quality Centre, 2001). The collection was obtained from raw milk tankers in which milk was plated onto Enterococcosel agar using a method developed by another CSIRO staff member (Helen Mitchell). The strains were confirmed as members of the genus *Enterococcus* using physiological and biochemical tests (Chapters 2.2.1 and 2.2.6.5) and
PCR (Chapter 6.2.3.1) and were speciated (Chapter 2.2.6.6). It was this collection of strains that was used to standardise procedures before undertaking the year-long survey. The standardisation consisted of two parts: firstly, determining which media gave the best selective isolation of enterococci species from mixtures of LAB and, secondly, what antibiotic resistance patterns were likely to be seen in the enterococci populations during the one-year survey. This set the experimental parameters used to enrich for antibiotic enterococci and confirmed the cut-off points for MICs for sensitive/resistant for new isolates. It was also advantageous that this collection was obtained at a similar time as the survey of antibiotic usage on dairy farms as antibiotic resistance in dairy enterococci could then be related to antibiotic usage.

The most frequently dispensed veterinary antibiotic was neomycin (Dairy Industry Quality Centre, 2001). The MIC levels detected for this antibiotic for the majority of *E. faecalis* strains in the collection were similar, with only a small proportion of strains having MIC levels greater than the majority of strains, corresponding with the MIC90 (Chapter 6). This suggests that resistance to neomycin was not prevalent and not influenced by antibiotic usage at that point in time. On the other hand, streptomycin and tetracycline were among the top six most often dispensed intramammary antibiotics (Dairy Industry Quality Centre, 2001) and resistance to these two antibiotics was prevalent in this collection. Furthermore, in the raw milk that was collected the following year, in 2002-2003, resistance to these antibiotics was widespread, indicating that resistances to these antibiotics were continuing in the dairy environment (Chapter 5). Analysis of the PFGE Types from the year-long survey showed that enterococci with resistances to erythromycin, tetracycline and streptomycin were also persisting in the raw milk silos at the factory that was studied in more detail. Persistence of certain PFGE Types may have arisen from two possibilities: enterococci populations persisting on farms that were being continually re-introduced into the silos over time, or PFGE Types remaining in the raw milk silos at the factory tested. The concurrence of strong biofilm ability in some of these PFGE Types suggests that at least some of these PFGE Types were persisting in the silos, as biofilms can enable bacteria to evade cleaning and thus persist (Knight, 2015). As this work was commenced a number of years ago and is the first of this type in Australia, it provides a baseline for enterococci knowledge in the Australian dairy industry.
A contemporary study of enterococci in the dairy industry could provide information on what has and has not changed since this collection of microorganisms was obtained, including changes in antibiotype and fingerprint of strains present.

**8.3 Streptomycin resistance**

Streptomycin resistance was a dominant antibiotic resistance present in the populations of both the collection of enterococci used for media, antibiotic resistance and virulence factor assessments (Chapters 2 and 6) as well as the enterococci persistent in raw milk silos (Chapter 5). The reason for streptomycin resistance in the majority of the enterococci assessed in the current work was unknown, as the ant(6')-Ia gene was detected in only a few of the isolates. *E. faecalis* 2004b, which was used in antibiotic resistance transfer experiments, did not amplify this gene but transconjugants of this isolate were able to acquire streptomycin resistance (Chapter 7). An attempt was made to determine if the ant(3")-Ia gene, the second aminoglycoside-modifying enzyme gene responsible for streptomycin resistance in enterococci (Chow, 2000), was present in *E. faecalis* 2004b using PCR (Clark et al., 1999). No PCR product was detected; however, as a positive control strain for the PCR was not available, the negative result could not be verified (results not presented). In addition to aminoglycoside-modifying enzymes, high-level streptomycin resistance can also be caused by ribosomal mutation and is thought to be a significant cause of streptomycin resistance in clinical enterococci (Eliopoulos et al., 1984). Streptomycin functions primarily by inhibiting protein synthesis by binding to ribosomes (Mingeot-Leclercq et al., 1999), which means that it is able to gain entry into enterococci cells to reach its target. Another possibility with regards to streptomycin resistance in enterococci is whether or not cell surface changes have occurred that result in excluding the antibiotic from the cells. In the enterococci persistent in the raw milk silos, there was a link with streptomycin resistance and biofilm formation (Chapter 5). Over 30 genes in enterococci have been reported to be associated with biofilm formation (Paganelli et al., 2012). A number of these genes encode proteinaceous adhesins, including asa1, responsible for AS, as well as esp and gelE, that are involved in the early stages of biofilm formation (Paganelli et al., 2012). Despite esp being present in 46 % of the enterococci strains in this study, 81 % of the strains with high-level streptomycin
resistance exhibited esp, indicative of a link with streptomycin resistance (Chapter 6). This raises the possibility that the proteinaceous adhesins produced by esp alter the enterococci cell wall in a way that may also facilitate entry of streptomycin into the cell and is an area for further investigation. The link between the surface structures of enterococci, biofilm formation and streptomycin resistance could be explored experimentally using a number of approaches, including gene knock-out of the esp gene and how this relates to the other two traits.

8.4 Overall risk

Dairy Australia recorded that 6800 million litres of milk were produced in Victoria over the year (2002-2003) that the present work was undertaken (S. Esposito, personal communication, 10-12 May 2005). Enterococci were detected in nearly all of the raw milk samples obtained, however their presence in the dairy products was much more limited (Chapter 2), showing that the majority of enterococci were reduced or eliminated following pasteurisation. Nonetheless, some strains of enterococci were thermoduric, particularly E. hirae, E. durans and E. faecium (Chapter 3) and were capable of growing in milk at refrigeration (4 °C) and elevated (7 °C) temperatures (Chapter 4). The risk is greater at 7 °C than at 4 °C, as the enterococci can reach significant counts (>10^7 cfu/mL) within three weeks at 7 °C, which is the potential shelf-life of hygienically packaged, good quality pasteurised milk (Deeth, 1986). This is also a temperature likely to be encountered in domestic refrigerators (Evans and Redmond, 2015). These strains were able to survive but not grow in yoghurt and cottage cheese (Chapter 4). These strains were screened for tetracycline and vancomycin resistance only (the first shown to have a high incidence in surveyed enterococci and the latter due to the importance of vancomycin resistance in nosocomial infections) and were sensitive to both. They were also screened for the virulence factors: none of the isolates (two each of E. faecalis, E. faecium, E. hirae and E. durans) contained any of the four virulence factors except for one E. faecalis (2356p1), which was haemolytic, exhibited gelatinase production and had the agg gene for AS. This shows that virulent strains of enterococci from dairy sources do have the potential to survive pasteurisation and persist in dairy products. Vancomycin-resistant enterococci have persisted in causing infection in Victoria over a sustained period (Johnson et al.,
2010; Coombs et al., 2014), spanning the time over which the enterococci in the current work were obtained; however no vancomycin-resistant *E. faecalis* or *E. faecium* were detected in the current work (Chapters 5 and 6). A Victorian study determined that resistance to vancomycin in enterococci was developed within the bowels of hospital patients during antibiotic therapy, indicating that the resistance is hospital-acquired and not community-acquired (Howden et al., 2013). Furthermore, the growth of enterococci in dairy products, when present, may be limited by the nature and storage conditions of the products, where pH, nutrients, carbon sources, water activity and organic acids may generate a hostile environment for enterococci, thereby decreasing the risk of food being a source of antibiotic-resistant enterococci.

### 8.5 Further research directions

The enterococci were speciated using a biochemical key (Manero and Blanch, 1999) that was current at the time of isolation of the enterococci. Since then further enterococci species have been identified (Chapter 1.1) (Franze et al. (2011). The *E. faecalis* in the selection of enterococci that were used for antibiotic resistance determinations were all confirmed as *E. faecalis* by PCR (Chapter 6.3.2). This provides confidence that other enterococci identified as *E. faecalis* using the biochemical key were actually *E. faecalis*. As the majority (74.3 %) of the isolates obtained in the raw milk survey were identified as *E. faecalis* (Chapter 2.3.5), the biochemical key was sufficient for the identification of the majority of isolates in this study. However, it is possible that some of the non-*E. faecalis* isolates speciated using the key may have been one of the species described after the biochemical key was developed. This has not proceeded due to funding constraints but it would be interesting to know if any of the isolates from this work belonged to the newer species or if novel species are present in the collection. Sequence analysis of PCR products from 16S RNA genes, and other genetic typing schemes, may also elucidate subtle genetic variations beyond PFGE typing of *E. faecalis* strains.

The mating procedures in a milk biofilm in the current study were a preliminary look at the possibility of transfer in such an environment, given that transfer between the strains occurred when they were filter-mated in broth individually at an optimal temperature of
37 °C (Chapter 7). Although the strains could grow in milk at the temperatures used (10 and 25 °C) and were recovered from biofilms after mating, further evidence could be obtained to support the lack of transfer in the milk biofilm by ascertaining if gene transfer occurred in filter mating mixtures of the strains and also filter mating in milk, in case the milk inhibited transfer. Bacterial biofilms are less likely to contain only a single species in the environment (Bremer et al., 2015); however, the strains in this study could be mated in the milk biofilm using individual recipients to ascertain if the recipients will mate in this environment without interference from other strains. Further experimental development is needed to demonstrate transfer in biofilms if this is, indeed, possible when this is known to be feasible in broth and filter matings.

As documented in Chapter 6, *E. hirae* appear to have inherent resistance to nitrofurantoin. The *E. hirae* were largely antibiotic-susceptible and possessed none of the virulence factors that were assessed in this work (Chapter 6). Lack of virulence in *E. hirae* has been suggested to correspond with the significant prevalence of CRISPR1-cas1 in *E. hirae*, which provides protection against bacteriophage and is inversely correlated with the ability to acquire genes such as those for antibiotic resistance (Lyons et al., 2015). Despite this, an *E. hirae* strain in the current study acquired *ermB* and *tetM* genes for erythromycin and tetracycline resistance, respectively, showing for the first time that antibiotic resistance transfer to *E. hirae* is possible, at least in the laboratory. Nitrofurantoin resistance was a feature of the small number of *E. hirae* strains (n = 8) assessed in this study, further pointing to some mechanism of inherent resistance. Further work could investigate, firstly, if the association with nitrofurantoin resistance was upheld in a greater number of more widely-sourced dairy enterococci and, secondly, in enterococci from other environmental, food and clinical sources. Determination of the mechanism of resistance could then be sought, to elucidate if the resistance is transferable to potential pathogens or if it is indeed inherent and not posing a risk of acquisition by other pathogens.

Outside of the current work, studies of enterococci in Australian dairy products have not been reported. Given that a thermoduric *E. faecalis* strain with multiple virulence factors was detected, it would be of interest to determine the presence of clinically-relevant antibiotic resistances in this and other widely-sourced strains of thermoduric dairy
enterococci. Although vancomycin-resistant enterococci in hospital settings may be hospital-acquired (Howden et al., 2013), such information is lacking for streptomycin-resistant enterococci in Australia and it is unknown if the potential ingestion of streptomycin-resistant enterococci may be connected to the high-level streptomycin-resistant enterococci which have been isolated from Victorian patients (Coombs et al., 2014). Streptomycin resistance was widely present in the enterococci in this study (Chapters 5 and 6). A Melbourne study found that the same strains of vancomycin-resistant enterococci can persist for year in patients who have had further antibiotic exposure (Karki et al., 2013), so it is possible that a similar phenomenon could happen with streptomycin-resistant enterococci, allowing comparison of current enterococci infections with food strains that have been obtained previously. It would be of benefit to establish if thermoduric dairy strains from the this study or a new study of more recently-obtained thermoduric dairy enterococci are genetically related to clinical enterococci in Australia to determine if dairy products are a reservoir for streptomycin-resistant enterococci.
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Prevalence, seasonality, and growth of enterococci in raw and pasteurized milk in Victoria, Australia

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ABSTRACT

This study investigated the prevalence, seasonality, and species variety of enterococci present in raw milk factory silos and pasteurized milk in 3 dairying regions in Victoria, Australia, over a 1-yr period. Additionally, the growth ability of thermoduric enterococci isolated in this study (Enterococcus faecalis, E. faecium, E. hirae, and E. durans) was determined in milk at temperatures likely to occur during storage, transport, and distribution, and before domestic consumption (4 and 7°C). Enterococci were detected in 96% of 211 raw milk samples, with an average count of 2.48 log10 cfu/mL. Counts were significantly lower in winter than summer (average 1.84 log10 cfu/mL) and were different between factories but not regions. Enterococcus faecalis was the most prevalent species isolated from raw milk in every factory, comprising between 61.5 and 83.5% of enterococcal species across each season. Enterococci were detected in lower numbers in pasteurized milk than in raw milk and were below the limit of detection on spread plates (<10 cfu/mL) after factory pasteurization. Residual viable cells were only detected following enrichment using 100-mL samples of milk, with 20.8% of the samples testing positive; this equated to a decrease in the average raw milk enterococci count of >4 log10 cfu/mL following pasteurization. Although E. faecalis predominated in raw milk and E. durans was found in only 2.9% of raw milk samples, E. durans was the most prevalent species detected in pasteurized milk. The detection of enterococci in the pasteurized milk did not correlate with higher enterococci counts in the raw milk. This suggested that the main enterococci populations in raw milk were heat-sensitive and that thermoduric enterococci survived pasteurization in a small numbers of instances. All of the thermoduric enterococci that were assessed for growth at likely refrigeration temperatures were able to grow at both 4 and 7°C in sterile milk, with generation times of 35 to 41 h and 16 to 22 h, respectively. Thermoduric enterococci were detected in pasteurized milk stored at 4°C for 2 wk (typically 1 to 9 cells/100 mL, up to 2.82 log10 cfu/mL), demonstrating the potential of enterococci to survive pasteurization and contribute to milk spoilage at refrigeration temperatures. This is particularly relevant for milk that is aseptically packaged to exclude gram-negative psychrotrophic bacteria and kept above the recommended storage temperature of ≤5°C.

Key words: Enterococcus, raw and pasteurized milk, dairy, season, growth

INTRODUCTION

Enterococci are widespread throughout the environment (Giraffa, 2002), are inhabitants of mammalian intestines (Stiles, 1989), and have been isolated from plants and soil (Franz et al., 1999). Enterococci have been isolated from dairy cow feces (Batish and Ranganathan, 1984; Gelsomino et al., 2001) but not always from every cow tested (Kagkli et al., 2007). The survival of enterococci in feces has been shown to be longer than that of other fecal indicators (Sinton et al., 2007). Enterococci, along with Escherichia coli, are used as indicators of fecal contamination in watercourses (Hampson et al., 2010), as well as in food (Stiles, 1989), and can serve as indicators of process hygiene and food and drinking water quality (Halkman and Halkman, 2014). Although enterococci are recognized as conferring beneficial properties to fermented milk products, if they are present in dairy foods after manufacture, they may pose food safety risks as potential pathogens and reservoirs of antibiotic resistance and contribute to food spoilage (Giraffa et al., 1997; Ogier and Serror, 2008; Franz et al., 2011). Consequently, the importance of identifying sources of enterococcal contamination in milk supplies and risk factors for and persistence in manufactured products has received increasing interest (for example, see Buhnik-Rosenblau et al., 2013; Hammad et al., 2015).
Enterococci can enter milk from human or animal feces, water sources, the farm environment, or from milking equipment, bulk storage tanks, and equipment used during milk harvesting or local processing (Gelsomino et al., 2001, 2002). The occurrence of enterococci in raw bovine milk has been explored in several countries, where Enterococcus faecalis was the predominant species found in studies from India, the Czech Republic, and Turkey (Batish and Ranganathan, 1984; Schlegelova et al., 2002; Citak et al., 2005), whereas Enterococcus faecium was the main raw milk species in a South African study (Wessels et al., 1988). In contrast, an Irish farmhouse study found that Enterococcus casseliflavus and Enterococcus faecalis were the most frequently isolated species in raw milk from a small herd of 27 cows (Gelsomino et al., 2001). These species were also found in human feces, cheese made from the raw milk, tap water, and milking equipment, whereas E. faecium dominated cow feces when isolated at all. Gelsomino et al. (2002) concluded that milking equipment was the probable source of milk contamination, where E. casseliflavus persisted in the machine and bulk tank on the one farm examined. The seasonality of occurrence of enterococci has not been investigated in milk and dairy products; however, a study has looked at the seasonality of bovine fecal indicator organisms, including enterococci, on New Zealand farms, over 1 yr (Sinton et al., 2007). That study found that although presumptive enterococci counts in feces were variable, the highest counts occurred in spring (Sinton et al., 2007), the peak milk harvesting season.

Although numerous studies have identified the presence of enterococci in raw and pasteurized bovine milk, no longitudinal studies on the ecology of enterococci in bovine milk have examined the seasonality of occurrence and changes of species over time. Seasonality is of particular interest to the dairy industry in Australia, as dairy production occurs in regions that vary significantly in maximum daily temperatures, climatic conditions (Australian Government, 2015), and nature of water supply. Victorian production of cow’s milk is 66% of national production (9.5 million tonnes), representing ~1.5% of world production (AHDB, 2015), and occurs in 3 major dairying regions to the north, east, and west of the capital city, Melbourne (Victorian Government, 2014). The northern (north of Melbourne) and southern (East and west of Melbourne) regions experience significant differences in monthly maximum and minimum temperatures (Supplemental Figures S1 and S2; http://dx.doi.org/10.3168/jds.2015-9335) and water supply varies from rain-fed only, irrigation, and ground water supplies (Victorian Government, 2014). Given that several factors may influence the nature of the microflora in raw milk (e.g., region, climate, water supply, transport distance to manufacturing plants, and temperature of transport), a survey was undertaken to determine whether seasonality, season of milk harvest (sampling over 1 yr), and location of manufacturing plants influenced the species type and prevalence of enterococci in raw milk as delivered to bulk holding facilities in manufacturing plants; to our knowledge, this is the first survey of this type in Australia. In addition, pasteurized milk from one manufacturing plant was tested over 1 yr immediately after manufacture, to determine the survival rate of enterococci after factory pasteurization (72°C, 15 s), and again after 2 wk of storage of milk at 4°C, to ascertain whether very low numbers of survivors could grow over a typical shelf-life period, and in line with the generation times measured for known thermoduric enterococci, to contribute to spoilage.

MATERIALS AND METHODS

Sample Acquisition

Raw bovine milk was sampled 12 times at approximately monthly intervals from 3 different bulk raw milk silos at each of 6 dairy factories in Victoria, Australia (n = 211). Dairy factories were located in Victoria’s 3 dairying regions, east (factories A and B), north (factories C and D), and west (factories E and F) of Melbourne, the capital of Victoria. All of the factories were within a 200-km radius of Melbourne and the distance between the centers of the regions was approximately 250 km. Samples were assigned to a season based on the month they were collected (summer: December, January, February; autumn: March, April, May; winter: June, July, August; spring: September, October, November). Milk was not collected from factory C in July (winter) and only 2 milk samples were collected from factory A in December (summer) and from factory C in February (summer). Factory-pasteurized milk (72°C, 15 s) from 2 processing lines at factory B was sampled monthly over a 12-mo period (n = 24). Milk samples were placed into insulated containers with either ice packs or crushed ice, transported to the CSIRO Melbourne laboratory by overnight courier and stored at <4°C. All testing was performed on the day of sample arrival.

Enumeration of Enterococci in Raw Milk

Chromocult Enterococci broth (CEB; Merck, Darmstadt, Germany), prepared as single-strength (1 × CEB) or double-strength (2 × CEB), and CEB with 15 g/L of Bacteriological Agar No. 1 (Oxoid, Basingstoke, UK; CEBA) were used in the detection of enterococci in the
raw and pasteurized milk. On arrival, raw milk samples were serially diluted in 0.1% Bacteriological Peptone (Oxoid; Standards Australia, 1991a; AS1766.1.2), and 0.1 mL (10⁰ to 10⁻² dilutions) was spread onto CEBAA, in duplicate (Standards Australia, 1991c; AS1766.1.4). All broths and plates were incubated at 37°C for 48 h. Presumptive enterococci colonies on the spread plates were enumerated (Standards Australia, 1991c; AS1766.1.4), counting all blue colonies with morphology typical of enterococci compared with the *E. faecalis* AS1766.1.4, counting all blue colonies with morphology typical of enterococci compared with the *E. faecalis* AS1766.1.4, counting all blue colonies with morphology typical of enterococci compared with the *E. faecalis* reference strain ATCC 29212. The limit of detection of enterococci by plate counts was 10 cfu/mL.

**Detection of Enterococci in Pasteurized Milk**

Pasteurized milk (0.1 mL) was spread in duplicate onto CEBAA without dilution, and then incubated and enumerated as above. Because numbers surviving factory pasteurization (72°C, 15 s) were expected to be low and potentially undetectable on plate counts, enrichment in CEB was undertaken using 3 inoculation strategies for all pasteurized milk samples, with the following ratios of milk to CEB: 1 mL into 10 mL of 1×CEB; 10 mL into 10 mL of 2×CEB; and 100 mL into 100 mL of 2×CEB. These enrichments had minimum detection levels of 1, 0.1, and 0.01 cfu/mL, respectively. All enrichment broths were streaked onto CEBAA plates to detect presumptive enterococci. The broth and plates were incubated at 37°C for 48 h.

**Selection of Isolates for Confirmation as Enterococci**

Representative isolates were obtained using a Harrison’s disc to randomly select colonies on spread plates (Harrigan and McCance, 1976), selecting the square root of the count on the plate. If the spread plates for pasteurized milk samples contained fewer than 20 enterococci colonies, 2 typical isolates were selected from the streak plates of the enrichment broths. All selected isolates were subcultured into 5 mL of 1×CEB and incubated at 37°C until turbid and the color of the broth had changed from clear yellowish to blue. The CEB cultures were then streaked onto brain-heart infusion agar (BHIA; Oxoid) plates and slopes, and incubated at 37°C for 18 to 24 h. Plates were checked for purity and colonies restreaked up to 2 times, if necessary, to obtain pure cultures.

**Confirmation of Isolates as Enterococci and Speciation**

All selected isolates from both the raw and pasteurized milk were confirmed as enterococci using tests for catalase reaction, pyrrolidonyl aminopeptidase (PYR) reaction, and growth at 45°C and in 6.5% NaCl (McAuley et al., 2012). Enterococci had a negative catalase reaction, determined by the lack of bubble formation when colonies were emulsified in 30% hydrogen peroxide (BDH, Kilsyth, Victoria, Australia). The PYR reaction was determined using Rosco Diatabs (Dutec Diagnostics, Croydon, NSW, Australia) added to a McFarland Standard 4 culture suspension in 0.85% saline, incubated at 37°C for 18 to 24 h. Enterococci were PYR positive, showing a red reaction. A McFarland Standard 4 culture suspension was spotted onto a BHIA agar plate and incubated at 45°C for 24 to 48 h. A 10-μL loop of the McFarland Standard 4 culture suspension was added to brain-heart infusion broth (BHIB; Oxoid) adjusted to 6.5% NaCl and incubated at 37°C for 24 to 48 h. Visible growth on the plate at 45°C and in 6.5% NaCl was characteristic of enterococci. The species of the confirmed enterococci were determined based on carbohydrate utilization in phenol red broth (Amyl Media, Dandenong, Victoria, Australia) using the method of Manero and Blanch (1999). *Enterococcus faecalis* ATCC 29212 was used as a positive control culture to verify the performance of all batches of media. It was also used as an example of *Enterococcus* in the confirmation tests. Confirmed cultures were stored on cryobeans (Microbank, Pro-Lab Diagnostics, Austin, TX) and in nutrient broth (Oxoid) with 20% glycerol (BDH) at −70°C.

**Growth of Enterococci in Inoculated Milk**

During the course of this survey, 71 raw milk samples from the 6 factories were subjected to laboratory pasteurization (63°C, 30 min), and a selection of surviving enterococci strains (8) was characterized in terms of species and thermotolerance (McAuley et al., 2012). The 8 strains comprised 2 isolates each of *E. faecalis*, 2350p1 and 2356p1; *E. faecium*, 2276p1 and 2299p2; *Enterococcus hirae*, 2239p2 and 2452p2; and *Enterococcus durans*, 2128p1 and 2151p1. These isolates were used in the work reported here to determine whether enterococci could grow in milk at typical transport and storage temperatures (4°C or 40°F; Clemson University, 2015; Dairy Food Safety Victoria, 2015; Government of Canada, 2015) or at abuse temperatures likely to be found in domestic refrigerators (7°C; Evans and Redmond, 2015).

The 8 isolates were individually inoculated into 10 mL of BHIB from BHIA slopes and grown for 20 h at 37°C. The BHIB cultures were diluted in 0.1% bacteriological peptone, and both isolates of each species were inoculated into UHT whole milk to obtain a starting count of 30 to 90 cfu/mL. The inoculated milk was dispensed in 10-mL volumes into multiple MacCartney
bottles for sampling at each time point and temperature. Milk incubated at 4°C was tested weekly for 5 wk. Milk incubated at 7°C was tested twice weekly for 3 wk as the growth was expected to be quicker. Total plate counts were obtained using BHIA pour plates of 1 mL of milk (Standards Australia, 1991b; AS1766.1.3) and BHIA spiral plates (Don Whitley Scientific, Shipley, UK), in duplicate. Plates were incubated at 37°C for 48 h and viable counts determined. Uninoculated UHT milk was incubated at each temperature and tested concurrently with the inoculated milk as a sterility control, using undiluted milk in pour plates. Growth rates were determined using counts transformed to log10 cfu/mL. The data were analyzed using the DMFit models on the ComBase website (http://modelling.combase.cc/membership/ComBaseLogin.aspx) using the Baranyi and Roberts (1994) model to determine initial and final counts, and maximum growth rate.

Detection of Enterococci During Storage of Pasteurized Milk

Following testing on arrival for plate counts or enrichment culture, the remaining pasteurized milk samples (n = 22) of approximately 1.9 L were stored at 4°C for 2 wk. The pasteurized milk was not inoculated with additional cultures and was assessed for the presence of naturally occurring enterococci at the end of shelf life (2 wk) at the legally required refrigeration temperature (≤5°C). After storage, the samples were spread plated (0.1 mL) onto CEBA and incubated at 37°C for 48 h. Samples were also enriched in CEB using 1-, 10-, and 100-mL volumes of milk to detect enterococci present in numbers below the limit of detection on spread plates. The enrichments were incubated at 37°C for 48 h and then streaked onto CEBA, which was incubated at 37°C for 48 h. A minimum of 2 presumptive enterococci isolates were taken from the streak plates of each positive sample and confirmed as described above. The species of the confirmed enterococci were determined as described above.

Data Analyses

Raw milk counts were transformed to log10 and analyzed using ANOVA to determine significant differences (P < 0.05) between the number of enterococci in samples from the factories, regions, and seasons (least significant differences, LSD) with Genstat Release 6.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, UK). The average number of enterococci in the raw milk was determined by calculating the geometric mean using a log10 transformation. Counts that were 0 on the plate (<10 cfu/mL) were analyzed as 1 cfu/mL. The prevalence of each species in the raw milk was determined by calculating the percentage of each species out of the total number of enterococci isolated. The ANOVA tables are located in Supplemental Tables S1 and S2 (http://dx.doi.org/10.3168/jds.2015-9335).

For the pasteurized milk enriched samples, results were recorded for the smallest sample size in which enterococci were detected; that is, presence in 1, 10, or 100 mL, or not detected in 100-mL enrichments, or counts in 0.1 mL (spread plates) in the case of 2-wk samples. The prevalence of each species of enterococci in the pasteurized milk was determined by calculating the percentage of samples containing each species.

RESULTS

Prevalence in Raw Milk

In total, 211 raw milk samples were collected from 6 milk processing plants in 3 dairy regions in Victoria over a 1-yr period, resulting in the acquisition of 909 isolates of enterococci. The data allowed analyses of the monthly incidence of enterococci at each factory (Figure 1), the range of counts seen across all of the factories, and variation in counts over 4 seasons across all of the factories (Figure 2). Enterococci were detected in 96% of the raw milk samples (detection limit 1 log10 cfu/mL), with counts ranging from <1 to 6.80 log10 cfu/mL (Figure 1) with an average of 2.48 log10 cfu/mL; most counts (77.3%) were <3 log10 cfu/mL. Factories A and F, which are located in the south of Victoria but in different regions, had the highest counts in individual samples (5 to >6 log10 cfu/mL).

The average counts of enterococci in raw milk from each factory throughout the year were all between 2 and 3 log10 cfu/mL; however, differences were found between factories (P = 0.003). Factory A had the highest enterococci counts (P = 0.003) compared with all of the other factories except factory F. The average number of enterococci was not different (P = 0.809) between the 3 regions (north, east, and west of Melbourne, Victoria).

A distinct decrease in counts (P = 0.008) was observed in winter (particularly July–August samples for most factories, Figure 1) relative to the other seasons when considering the data across the 3 regions (Figure 2), with the winter counts being 0.64 log10 cfu/mL lower compared with those in summer.

Enterococci Species in Raw Milk

A total of 909 isolates were confirmed as enterococci and analyses of species type in all of the raw milk
samples determined which species were predominant (frequency of isolation), the mix of species at each factory (Figure 3a), and seasonal variation in species type (Figure 3b).

The following 13 Enterococcus species were detected in the 211 raw milk samples (% of total isolates): E. faecalis (74.3%), E. faecium (8.6%), E. hirae (8.5%), E. casseliflavus (3.5%), E. durans (2.9%), E. asini (1.1%),

Figure 1. The monthly average raw milk counts of enterococci for 6 dairy factories. SED = standard error of difference of means.
Enterococcus faecalis, E. faecium, and E. hirae were the predominant species (defined as a species present in more than 50% of the samples) in 72.9, 6.3, and 4.2% of the raw milk samples with counts of ≥3 log_{10} cfu/mL, respectively. The remaining 8 samples (16.6%) did not contain a predominant species. Four milk samples had enterococci counts of 5 log_{10} cfu/mL or greater, with E. faecalis being predominant, and E. faecium was predominant in only 1 sample.

The most prevalent Enterococcus species detected in each factory was E. faecalis (61.2 to 88.5%; Figure 3a). The greatest occurrence of E. faecalis was in factory A, whereas factory F had the least. Conversely, factory F had the most E. faecium, whereas factory A had the least. Notably, these 2 factories had different microflora from the other factories and also yielded samples with the 4 highest counts.

Seasonal differences for E. faecalis and E. faecium (Figure 3b) were observed, with the prevalence of E. faecalis increasing in summer and that of E. faecium in autumn. Enterococcus faecalis remained the most prevalent species isolated in every season, including spring, the peak milking season (Dairy Australia, 2014). Seasonal distributions for E. faecium and E. casseliflavus were similar to each other, although numbers were lower for the latter species.

**Prevalence in Pasteurized Milk**

Enterococci counts from pasteurized milk following manufacture at factory B were below the limit of detection on spread plates (<1 log_{10} cfu/mL), and enterococci were detected in only 5 (20.8%) of the 24 samples following enrichments using 100 mL of milk in 2×CEB. Because the limit of detection in the 100-mL enrichments was 0.01 cfu/mL, assuming a minimum number in the 100-mL volume, this represented a calculated decrease in the average raw milk enterococci count (2.48 log_{10} cfu/mL) of approximately 4 log_{10} cfu/mL. Three of the 5 positive samples were obtained in the autumn and 1 each in winter and summer; none were detected in spring (Table 1). The highest raw milk counts in samples from factory B were 3 to 4 log_{10} cfu/mL in spring (P = 0.027), so that persistence after pasteurization did not correlate with higher initial bacterial loading in raw milk.

Enterococcus durans was detected at the highest frequency among pasteurized milk samples (4/5) in the summer and autumn; the remaining positive sample contained E. malodoratus (1/5) in the winter (Table 1); these species were not predominant in raw milk samples from this factory (Figure 3a).

**Growth of Enterococci in Inoculated Milk**

Figure 4 shows the growth rates of E. faecalis, E. faecium, E. hirae, and E. durans isolates (McAuley et
al., 2012) when cultured in UHT milk at 4 and 7°C with inocula containing 2 strains of the same species. The viable count numbers represent the total for both strains in the one culture. The lag phase was longer for all isolates at 4°C, with viable counts of *E. faecalis*, *E. faecium*, and *E. durans* remaining unchanged after the first week at 4°C and only increasing by 0.5 to 1.0 log_{10} cfu/mL after 2 wk. Because the stationary phase did not appear to be reached for *E. faecalis*, *E. faecium*, and *E. durans* at 4°C, there was potential for further growth of these isolates if the incubation period had continued. At 7°C, all cultures achieved a maximum viable count between 7.4 and 8.1 log_{10} cfu/mL within 3 wk. The generation times for the species tested were 35.5 to 41.0 h at 4°C and 15.9 to 22.0 h at 7°C (Table 2).

![Statistical chart](chart.png)

**Figure 3.** Percentage of the 5 main *Enterococcus* species from raw milk (a) at each factory averaged across all samples, and (b) in each season averaged across all factories.
Detection of Enterococci During Storage of Pasteurized Milk

Twenty-two of the 24 of pasteurized milk samples were stored for 2 wk at 4°C and then tested using plate count and enrichment procedures to detect enterococci potentially present initially at levels of <1 cfu/100 mL. Calculations, based on the lowest growth rate of the thermoduric enterococci isolated in this study (Table 2), indicated that the 2-wk storage period provided sufficient generation times to enable detection of enterococci at a level of at least 1 cfu/100 mL (and even as low as 1 cfu/L).

Three of the 22 pasteurized milk samples had detectable enterococci initially. Enterococci were detected in 40.9% of pasteurized milk samples after 2 wk of storage.
(Table 1). The majority of these detections occurred in 100-mL enrichments (22.7%); however, enterococci were detected in 4 enrichments of <100 mL. One sample had a measurable count of 2.84 log_{10} cfu/mL on a CEBA spread plate, where the prevalent species, *E. durans*, was the main species found in milk immediately after pasteurization (Table 1). This level of viable count is consistent with the measured generation time for this species (Figure 4, Table 2), the starting count of 0.01 to 0.09 log_{10} cfu/mL, and incubation period of 2 wk at 4°C. Enterococci were not detected in 54.5% of the samples either following manufacture or after 2 wk of storage, as they were below the limit of detection (<1 cfu in 100 mL).

Immediately after factory pasteurization, *E. durans* and *E. malodoratus* were detected at 0.01 to 0.09 cfu/mL, whereas after storage at 4°C for 2 wk, 4 other species in addition to *E. durans* (*E. faecalis, E. hirae, E. asini,* and *E. sulfureus*) were detected in 9 samples and *E. malodoratus* was not detected. This indicated that very low numbers of several thermoduric enterococci had survived pasteurization and were able to grow in milk at 4°C to detectable numbers. The 10- and 1-mL enrichments contained *E. asini* and the 0.1-mL spread plate contained *E. durans*; *E. faecium* was not detected in the pasteurized milk initially or after storage.

**DISCUSSION**

Enterococci are commonly found in raw milk and processed products, with different flora reported in different countries, reflecting local practices and levels of hygiene (Batish and Ranganathan, 1984; Wessels et al., 1988; Citak et al., 2005). It is generally accepted that farms and manufacturing facilities may develop a specific microflora, giving rise to typical product traits that make these distinctive or regionally recognizable (Giraffa, 2002; Quigley et al., 2011). Sources tracking entry of enterococci into raw milk and subsequent transmission into processed products have indicated persistence of particular species or strain types, where the likely source of reintroduction of these is through milk harvesting and processing equipment, as well as through common flora residing in workers involved in the supply chain (Gelsomino et al., 2001, 2002), although it is not clear whether enterococci originate in cows or humans and are subsequently shared microflora (Gelsomino et al., 2002; Kagkli et al., 2007). Consequently, at the outset of the survey, we anticipated that the enterococcal microflora may vary between dairying regions and over time in Victoria due to influences including climate and seasonal temperature differences, water supply, the specific microflora in farms and processing plants servicing the regions, and the transport systems operating, given that prior studies have indicated the diversity of enterococci found in particular farming and manufacturing settings (Cogan et al., 1997; Franciosi et al., 2009; Jackson et al., 2011). The centers of the regions in the current study are approximately 250 km apart from each other with milk collection within the region and thus may represent potentially distinct pools of microbes that have evolved in the particular supply systems around the processing plants. Transport from farms involves varying distances so the critical point for determining raw milk quality is in bulk storage before processing, which provided an overview of microbial quality in terms of enterococci count. The data collected over this 1-yr survey indicated significant differences between individual factories. However, factory location in the state did not correlate with differences in incidence, species, or counts, despite differences in climatic conditions between the regions. Temperature differences in summer were not an important factor, which implies good practice in milk collection and transport and compliance with regulated temperature control requirements. Milk must be chilled to ≤5°C within 3.5 h of the start of milking and transported at ≤5°C; it is the manufacturer’s responsibility to ensure valida-

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<th>Species</th>
<th>Incubation temperature (°C)</th>
<th>Initial value (^1) (log(_{10}) cfu/mL)</th>
<th>Final value (^1) (log(_{10}) cfu/mL)</th>
<th>Maximum rate (^2) (log(_{10}) cfu/mL per hour) × 100</th>
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<td>1.68</td>
<td>6.42</td>
<td>0.73</td>
<td>41.0</td>
</tr>
<tr>
<td><em>E. hirae</em></td>
<td></td>
<td>1.48</td>
<td>7.10</td>
<td>0.79</td>
<td>37.6</td>
</tr>
<tr>
<td><em>E. durans</em></td>
<td></td>
<td>1.47</td>
<td>6.76</td>
<td>0.85</td>
<td>35.5</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>7</td>
<td>1.75</td>
<td>7.41</td>
<td>1.91</td>
<td>15.9</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td></td>
<td>1.82</td>
<td>8.10</td>
<td>1.48</td>
<td>20.8</td>
</tr>
<tr>
<td><em>E. hirae</em></td>
<td></td>
<td>1.96</td>
<td>7.67</td>
<td>1.37</td>
<td>22.0</td>
</tr>
<tr>
<td><em>E. durans</em></td>
<td></td>
<td>1.51</td>
<td>8.01</td>
<td>1.65</td>
<td>19.5</td>
</tr>
</tbody>
</table>

\(^1\)Inoculated level.

\(^2\)All curves had R-values >0.99.
tion of temperature control procedures and equivalence demonstrated to ensure minimization of pathogenic organism growth (Dairy Food Safety Victoria, 2015). Significant differences in load and species type were observed between factories, suggesting that hygiene or management practices in supply and storage need to be addressed, and this factor was more important than regional variation. Distinctive enterococcal flora were observed between the factories but *E. faecalis* dominated in all factories. *Enterococcus faecium* was also found in all of the factories, and was more predominant in 1 factory (factory F), which had a decreased presence of *E. faecalis*.

Seasonality was observed when data from all samples taken from all factories were pooled. Lower counts were obtained in winter and the ratio of species mixes also differed in every season. *Enterococcus faecium* was generally proportionately higher when the incidence of *E. faecalis* was lower, although *E. faecalis* dominated all samples. This was similar to the dominance of this species in other reports (Batish and Ranganathan, 1984; Schlegelova et al., 2002; Citak et al., 2005).

We observed a low prevalence of the more thermoduric species in the raw milk (*E. faecium*, *E. hirae*, and *E. durans*). Survival of a few enterococci occurred at a detection level of 0.01 to 0.09 log_{10} cfu/mL after pasteurization (72°C, 15 s), with *E. durans* being most prevalent. *Enterococcus durans* was one of the more thermophilic species assessed in pasteurized milk, whereas *E. faecalis* was comparatively more heat-sensitive (McAuley et al., 2012).

Limited consortia of 4 species that had previously been shown to be heat resistant (McAuley et al., 2012) showed that these species could grow at 4°C and at the elevated refrigeration temperatures (7°C) likely to be encountered in domestic fridges (Evans and Redmond, 2015). Growth at 10°C is one of the defining characteristics of the original description of the *Enterococcus* genus (Devriese and Pot, 1995) but growth at 4 to 7°C has not been characterized previously. Growth kinetics showed a lag period for some cultures and some variation in growth rate, with the growth rate being significantly slower at 4°C than at 7°C. However, the species that survived pasteurization at <1 cfu/100 mL included *E. durans* as well as several other species. Knowing the approximate doubling time of the species at 4°C was useful in understanding the incubation time needed to detect very small numbers of survivors of pasteurization—0.01 to 0.09 cells/mL. Understanding the growth kinetics of thermophilic enterococci for the species not investigated here may inform the setting of shelf-life periods to minimize spoilage. Further work could be undertaken to understand the growth kinetics of other thermophilic enterococci.

Enrichment strategies for detecting very low numbers of survivors or damaged cells were useful, albeit semiquantitative. This allowed the order of magnitude of contaminants to be determined and facilitated detection and identification of the thermophilic species surviving pasteurization. Enrichment enabled the detection of the range of species that survived pasteurization, which would have been otherwise undetected by standard plating methods. Survival of *Enterococcus* species in pasteurized milk is important in terms of the ability to grow subsequently and contribute to spoilage or pose health risks: this study showed that it is important to use enrichment methods to detect and identify the species that survive in low numbers.

**CONCLUSIONS**

A survey of enterococci in raw milk from factory silos demonstrated that these bacteria are widespread in milk before manufacture. The occasional occurrence of high levels of enterococci in a small number of samples suggests that environmental contamination is at times high and hygiene of milk collection and in factories needs to be improved in some instances. Seasonal differences were observed, with lower counts occurring in winter; this has not been reported in other studies of bovine raw milk conducted internationally. Enterococci were able to survive and grow at 4°C in naturally contaminated pasteurized milk. *Enterococcus durans* was detected in the greatest number of pasteurized milk samples after manufacture, although *E. faecalis* was the predominant species detected in raw milk. The level of detection of enterococci in pasteurized milk was low and did not coincide with higher raw milk counts, indicating that the main enterococci population was heat-sensitive. Thermophilic enterococci were able to grow at 4 and 7°C in UHT milk. When milk inoculated with 10^2 cfu/mL was stored at 7°C, enterococci numbers reached spoilage levels in 2 wk. This demonstrates the potential ability of enterococci that can survive pasteurization to be spoilage bacteria if milk is stored above the recommended refrigeration temperature of 4°C.

**ACKNOWLEDGMENTS**

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analysis of results of the survey to determine the prevalence of enterococci in the Australian dairy industry. Technical assistance by Marie Collier (CSIRO, Werribee, Victoria) is also gratefully acknowledged.

REFERENCES


Heat resistance of thermoduric enterococci isolated from milk

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A B S T R A C T
Enterococci are reported to survive pasteurisation but the extent of their survival is unclear. Sixty-one thermoduric enterococci isolates were selected from laboratory pasteurised milk obtained from silos in six dairy factories. The isolates were screened to determine log10 reductions incurred after pasteurisation (63 °C/30 min) and ranked from highest to lowest log10 reduction. Two isolates each of Enterococcus faecalis, Enterococcus faecium, Enterococcus durans and Enterococcus hirae, exhibiting the median and the greatest heat resistance, as well as E. faecalis ATCC 19433, were selected for further heat resistance determinations using an immersed coil apparatus. D values were calculated from survival curves plotted from viable counts obtained after heating isolates in Brain Heart Infusion Broth at 63, 69, 72, 75 and 78 °C followed by rapid cooling. At 72 °C, the temperature employed for High Temperature Short Time (HTST) pasteurisation (72 °C/15 s), the D values extended from 0.3 min to 5.1 min, depending on the isolate and species. These data were used to calculate z values, which ranged from 5.0 to 9.8 °C. The most heat sensitive isolates were E. faecalis (z values 5.0, 5.7 and 7.5 °C), while the most heat resistant isolates were E. durans (z values 8.7 and 8.8 °C), E. faecium (z value 9.0 °C) and E. hirae (z values 8.5 and 9.8 °C). The data show that heat resistance in enterococci is highly variable.

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1. Introduction
Pasteurisation of milk is applied primarily to eliminate the presence of pathogenic microorganisms although it will also reduce the levels of spoilage bacteria in raw milk (Holsinger et al., 1997; Lewis, 2003). A typical high temperature-short time (HTST) pasteurisation treatment of raw milk involves rapidly heating the milk to a minimum of 72 °C for 15 s, followed by rapid cooling to 4.5 °C (Lewis, 2003; FSANZ, 2004). Pathogens such as Campylobacter and Salmonella can undergo a 5–8 log10 reduction with such a treatment (Lewis, 2003). Throughout history, pasteurisation conditions have been modified as new information about the heat resistance of target microorganisms has been identified (Holsinger et al., 1997), and it is important that legislative requirements and food processors keep up with new information as it becomes known.

Enterococci may enter raw milk and dairy products during manufacture from human, animal or environmental sources and have been suggested as useful indicator organisms for process hygiene (Garg and Mital, 1991; Giraffa, 2003). Several authors have alluded to the heat resistance of enterococci (Franz et al., 1997; Giraffa, 2002; Martinez et al., 2003) and it has been noted that enterococci can survive pasteurisation temperatures (Giraffa et al., 1997; Rao et al., 1986) but the extent of their survival is unclear. Although enterococci are potentially beneficial in some dairy products as starter or probiotic cultures (Giraffa, 2003), they are also considered to be potential spoilage microorganisms and may present a health risk to humans through the production of biogenic amines during cheese manufacture (Garg and Mital, 1991). Furthermore, isolates of some species of enterococci are opportunistic pathogens with intrinsic or acquired antibiotic resistance which may be transferred to other pathogens on conjugative transposons and conjugal and non-conjugative plasmids (Franz et al., 1999) and it is generally undesirable to have such microorganisms in food. The purpose of this work was to determine the effect of pasteurisation conditions on the survival of heat resistant enterococci and to obtain data that would allow the prediction of the thermal treatments required to inactive populations of thermoduric enterococci which may be found in raw milk.

2. Materials and methods
2.1. Strains and culture maintenance

Raw milk was collected monthly from the refrigerated bulk milk silos at six dairy factories (A, B, C, D, E and F) in Victoria, Australia, over 1 year. Three milk samples were obtained per factory in each season except for Factory C which only provided two samples in the
winter. Laboratory pasteurisation of 100 mL of 71 raw milk samples was performed at 63 °C/30 min, followed by rapid cooling. Surviving enterococci were selected from plates containing Chromocult Enterococci broth. The identity of the resulting 176 isolates was confirmed using biochemical tests (catalase negative, pyrrolidonyl aminopeptidase positive and growth in 6.5% NaCl and at 45 °C). Isolates were speciated according to the method of Manero and Blanch (1999).

One isolate each of the four predominating species was chosen from each factory and season for further analysis, selecting from all three milk samples in a season, where possible. The Enterococcus faecalis ATCC 19433 Type strain (reported as NCTC 775) has been used to validate date thermal inactivation processes (Bradley and Fraise, 1996). It was used as a control in this study for comparative purposes in the immersed coil procedure and was obtained from bioMérieux (Marcy l’Etoile, France).

Enterococci isolates were grown in Brain Heart Infusion (BHI, Oxoid) broth (37 °C/18–24 h) and inoculated onto BHI agar slopes (37 °C/18–24 h). Working cultures were obtained from the agar slopes. Cultures were stored long-term on cryobeads (Microbank™, Pro-Lab Diagnostics, Austin, Texas, USA) at −70 °C.

2.2. Thermotolerance ranking

The isolates were grown for 20 h in BHI broth, diluted to 10^7 cfu/mL in 10 mL of BHI broth and pasteurised in the laboratory. The pre- and post-pasteurisation samples were spiral plated (Don Whitley Scientific Limited, Shipley, West Yorkshire, England), in duplicate, onto BHI agar plates. Post-pasteurisation samples were also poured plated in duplicate (1 mL in BHI agar) (Standards Australia, 1991). The plates were incubated at 37 °C for 72 h and the colonies were counted. Log_{10} reductions were calculated by subtracting the log_{10} cfu/mL post-pasteurisation count from the log_{10} cfu/mL pre-pasteurisation count. The results were used to rank the isolates from each species into the order of highest to lowest log_{10} reduction after pasteurisation. For each of the four species, one isolate with the median heat resistance and one isolate with the greatest heat resistance were selected to represent typical and more highly heat resistant enterococci, respectively.

2.3. Thermal death determinations

2.3.1. Immersed coil procedure

The eight selected pasteurised milk isolates (Table 1) and E. faecalis ATCC 19433 were tested for their survival at 63, 69, 72, 75, 78 and 81 °C using an immersed coil apparatus (Sherwood Instruments, Lynnfield MA, USA). The isolates were inoculated individually into 10 mL of BHI broth and incubated at 37 °C for 20 h. The concentration of cells was adjusted to between 10^7 and 10^8 cfu/mL with BHI broth and 10 mL of the diluted culture was injected into the immersed coil using a sterile syringe (Terumo, Binan, Laguna, Philippines). The coil instantaneously heated the culture to the required temperature and discharged 400 μL at ten equidistant time periods into 3.6 mL of chilled 0.1% Bacteriological Peptone (Oxoid). Samples were immediately placed in ice water, ensuring rapid cooling. The samples, before and after heating, were serial diluted as necessary. Plates were incubated at 37 °C for 72 h before counting.

2.3.2. Optimum incubation time

The optimum incubation period for recovering surviving, possibly heat damaged, cells on plates was determined by measuring the recovery of Enterococcus durans 2151p1 and E. faecalis 2350p1 after heat treatment at 75 °C. After 75 °C heat treatment, the plates for these two isolates were incubated at 37 °C for 24, 48, 72 and 96 h. The counts were analysed using analysis of variance to determine the optimum incubation time after which additional incubation did not result in the detection of further colonies.

2.3.3. Calculations

Thermotolerance was determined once for all isolates by calculating the reduction in viability, in log_{10} cfu/mL, after laboratory pasteurisation. Standard deviations were calculated for each species (Microsoft® Excel 2003). For the thermal death determinations using the immersed heating coil, survivors of each isolate (log_{10} cfu/mL) were plotted against time using single determinations at each temperature, generating scatter graphs using Microsoft Excel. The D values (time required for counts to decrease by one log_{10} cfu/mL) were calculated from the resulting linear regression equations on the linear portion of the curves. Two thermal death curves were generated for all of the isolates, except 2356p1, at the key temperature of 72 °C. Statistical differences between the D values for two independent experiments were analysed using analysis of variance. Analysis of variance was conducted using GenStat Release 13.1 (VSN International Ltd., Hemel Hempstead, England). The log_{10} D values were plotted against the temperature at which the D value was determined for four temperatures (selected from 63, 69, 72 and 78 °C) on X-Y scatter graphs. Linear regressions were performed for each isolate to calculate the z values (decrease in D value by one log_{10} unit).

The Weibull model was originally developed for application to non-linear thermal inactivation data (Peleg and Cole, 1998). In this study, the model (LogS = −b + 10^n) was used to calculate the log_{10} reductions at 72 °C for 15 s, where LogS is the log_{10} reduction in the population of microorganisms, t is the time, and b and n are constants for the scale and shape parameters, respectively (Mattick et al., 2001). Additionally, this model was used to calculate the time required to achieve a six log_{10} reduction at 72 °C.

3. Results

3.1. Thermotolerance ranking

Between 9 and 12 enterococci isolates were tested from each of six factories for their relative ability to survive laboratory pasteurisation. E. faecalis, Enterococcus faecium, E. durans and Enterococcus hirae were represented in all of the factories, except Factory D, in which E. faecalis was not isolated from the laboratory pasteurised milk. The range of log_{10} reductions achieved by laboratory pasteurisation for each species was 3.5 to 0.3 log_{10} cfu/mL for E. faecalis (Fig. 1a). 3.2 to 0.1 log_{10} cfu/mL for E. faecium (Fig. 1b). 2.7 to 0.4 log_{10} cfu/mL for E. durans (Fig. 1c) and 4.0 to 0.4 log_{10} cfu/mL for E. hirae (Fig. 1d).

Table 1

Enterococci isolates used in thermal death determinations.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Species</th>
<th>Factory</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 19433</td>
<td>E. faecalis</td>
<td>N/A</td>
</tr>
<tr>
<td>2350p1</td>
<td>E. faecalis</td>
<td>A</td>
</tr>
<tr>
<td>2356p1</td>
<td>E. faecalis</td>
<td>F</td>
</tr>
<tr>
<td>2276p1</td>
<td>E. faecium</td>
<td>C</td>
</tr>
<tr>
<td>2209p2</td>
<td>E. faecium</td>
<td>F</td>
</tr>
<tr>
<td>2128p1</td>
<td>E. durans</td>
<td>D</td>
</tr>
<tr>
<td>2151p1</td>
<td>E. durans</td>
<td>B</td>
</tr>
<tr>
<td>2230p2</td>
<td>E. hirae</td>
<td>A</td>
</tr>
<tr>
<td>2452p2</td>
<td>E. hirae</td>
<td>E</td>
</tr>
</tbody>
</table>
When the holding period was extended beyond 10 s, the counts were significantly lower compared with 24 h incubation after the first 10 s holding period. From these results, one isolate with greater heat resistance and median heat resistance were selected for each species to evaluate D and Z values. The selected isolates were E. faecalis 2350p1 and 2356p1, E. faecium 2276p1 and 2299p2, E. durans 2128p1 and 2151p1 and E. hirae 2239p2 and 2452p2.

### 3.2. Thermal death determinations

#### 3.2.1. Incubation time

To determine the incubation time required to recover heat-stressed or damaged cells, two isolates were evaluated in the immersed coil apparatus at 75 °C for up to 5.25 min. For incubation of 24, 48, 72 and 96 h of incubation at 37 °C. Two-way analysis of variance for E. faecalis 2151p1 and E. faecium 2276p1 and 2299p2, E. durans 2128p1 and 2151p1 and E. hirae 2239p2 and 2452p2.

#### 3.2.2. Survival curves

The prepared cultures were each heated at five temperatures between 63 and 81 °C and samples were taken for up to 10 time periods, not including the unheated control. Time periods varied depending on the heating temperature, with intervals of 5 to 10 min at the lowest temperature and 7 to 10 s at the highest temperature. Survival curves were constructed to assist in the calculation of D values for each isolate.

### Table 2

<table>
<thead>
<tr>
<th>Holding period at 75 °C</th>
<th>Counts (log_{10} cfu/mL) after increasing incubation times</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 s</td>
<td>7.86&lt;sup&gt;a&lt;/sup&gt; 7.86&lt;sup&gt;a&lt;/sup&gt; 7.86&lt;sup&gt;a&lt;/sup&gt; 7.86&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 s</td>
<td>6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>20 s</td>
<td>6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>30 s</td>
<td>6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>40 s</td>
<td>6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>50 s</td>
<td>6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>60 s</td>
<td>6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>70 s</td>
<td>6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>80 s</td>
<td>6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>90 s</td>
<td>6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>100 s</td>
<td>6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt; 6.62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Different letters indicate significant differences (p < 0.001) within a row, lsd = 0.2394.
each isolate. The survival curves at 63 °C had an average reduction of approximately 0.5 log₁₀ cfu/mL after 30 min for all of the pasteurised milk isolates (Fig. 2a). This was markedly different from the E. faecalis ATCC 19433 Type strain, which had a 3.8 log₁₀ cfu/mL decrease in this time. E. faecalis 2350p1 and E. faecium 2276p1 had 1.9 and 2.1 log₁₀ cfu/mL reductions, respectively, after 100 min, whereas E. hirae 2452p2 and E. faecalis 2356p1 incurred the greatest reductions which were 3.9 and 4.4 log₁₀ cfu/mL, respectively. At 72 °C, E. faecalis ATCC 19433 was again the least heat resistant (Fig. 2b), sustaining a 3.5 log₁₀ cfu/mL decrease after 15 s. With the exception of one other E. faecalis isolate (2356p1), the majority of the pasteurised milk isolates did not show a similar reduction in counts until after 5 min at 72 °C.

The higher temperatures of 75 °C (Fig. 2c), 78 °C (Fig. 2d) and 81 °C were assessed for six of the isolates (2128p1, 2151p1, 2276p1, 2299p2, 2350p1, 2452p2) which showed greater thermal resistance. All of the pasteurised milk isolates were viable after 1 min at 75 °C, with reductions ranging from 5.0 log₁₀ cfu/mL for the most sensitive isolate (E. faecalis 2356p1) to 0.8 log₁₀ cfu/mL for the most heat resistant isolate (E. hirae 2452p2). At 78 °C, the two most heat sensitive pasteurised milk isolates were not detectable past the first time point of 10 s, while the remaining six pasteurised milk isolates had reductions ranging from 0.8 log₁₀ cfu/mL (E. faecalis 2350p1) to 2.9 log₁₀ cfu/mL (E. faecium 2276p1) after 10 s. Notably, these isolates were more similar after 100 s with reductions of 4.3 and 4.9 log₁₀ cfu/mL, respectively. None of these isolates had detectable counts after 10 s at 81 °C (detection limit of 10² cfu/mL). For the two most heat sensitive isolates, E. faecalis 2356p1 and E. hirae 2239p2, survival was additionally assessed at 69 °C to provide a fourth temperature with which to generate D values, since the generation of a survival curve was not possible at 78 °C. As E. faecalis ATCC 19433 was also rapidly killed at 75 °C, it was assessed at 63, 66, 69 and 72 °C. At 69 °C, E. faecalis 2356p1 and E. hirae 2239p2 incurred reductions of 4.3 and 0.5 log₁₀ cfu/mL after 5 min, but E. faecalis ATCC 19433 had already been reduced by 4.1 log₁₀ cfu/mL after 2.5 min.

### 3.2.3. D values

The D values decreased with increases in temperature as expected for all of the isolates, but the rate of decrease was different among isolates. For example, although E. faecalis 2350p1 had a much higher D value at 63 °C than all of the other isolates tested (Table 3), at 78 °C it was almost the same as the other isolates, which all had D values of 0.4 or 0.5 min. E. hirae 2452p2 and E. hirae 2239p2 had comparable D values at 63 °C but their calculated heat resistance at the higher temperatures was markedly different. While E. hirae 2239p2 could not be detected after the first time point at 78 °C and was only tested at a maximum of 75 °C, E. hirae 2452p2 could be assessed at 78 °C. Heat resistance at lower temperatures was not necessarily an indication of resistance at the higher temperatures. However, the Type strain E. faecalis ATCC 19433, which had the lowest D value at 63 °C, also proved to be less tolerant than any of the other isolates at the higher temperatures. It had a D value of 0.5 min at the additional temperature of 66 °C. The D value could not be calculated for this isolate at 72 °C using linear regression as the curve tailed after the second time point leaving only the first two points from which a robust D value could not be calculated. This situation also occurred for E. faecium 2276p1 at 78 °C. Analysis of variance of the D values showed that there

![Fig. 2. Survival of nine enterococci isolates at a) 63 °C, b) 72 °C, c) 75 °C and d) 78 °C using the immersed heating coil. The time scale varies between the temperatures to illustrate the characteristics of the curves.](image-url)
were no significant differences ($p > 0.05$) between the isolates or between the species at 72 °C.

3.2.4. $z$ values

When the log$_{10}$ D values were plotted against temperature, the fitted response line had good correlation coefficients ($R$) which were over 0.9 for all of the graphs. For the enterococci tested in this study, $z$ values ranged from 5.0 to 9.8 °C (Table 4). The isolates with the lowest $z$ values were all *E. faecalis*. Despite being less heat sensitive at the higher temperatures of 75 and 78 °C, *E. faecalis* 2356p1 from pasteurised milk had similar $z$ values to the *E. faecalis* Type strain. The isolates showing the greatest heat resistance belonged to the species *E. faecium*, *E. durans* and *E. hirae*.

3.2.5. Weibull model

Not all of the survival curves in the present study were linear, hence the Weibull model was applied to better interpret the data. Log$_{10}$ reductions of the enterococci at 72 °C after 15 s were predicted using both the D value curves and the Weibull model (Table 4). They were generally predicted to be greater using the Weibull model, ranging from 0.10 to 3.33 log$_{10}$ cfu/mL compared with 0.05 to 0.79 log$_{10}$ cfu/mL using linear regression. When the model was used to predict the time required to obtain a 6 log$_{10}$ cfu/mL reduction at 72 °C, the times ranged from 6 to 34 min. The isolates that reached a 6 log$_{10}$ cfu/mL reduction the fastest did not necessarily have the greatest reductions after 15 s. For instance, *E. faecium* 2276p1 took the longest to reach a 6 log$_{10}$ cfu/mL reduction but the reduction (Weibull) after 15 s was mid-range.

4. Discussion

When the thermopic enterococci isolates were screened individually in BHI broth for their ability to survive batch pasteurisation (thermotolerance ranking), the average log$_{10}$ reduction of 61 *E. faecalis*, *E. faecium*, *E. durans* and *E. hirae* isolates was 1.1 log$_{10}$ cfu/mL, with the most resistant isolates belonging to *E. durans*. In addition to heat resistance being influenced by species, it was also influenced by location. The majority of heat resistant *E. faecium* isolates were from one factory while the majority of heat sensitive isolates in general were from two other factories. Possible reasons for these variations may be due to different selective influences in the original environments, including exposure to different chemicals (including cleaning agents), heat and cold, all of which may have influenced thermal tolerance (Fernández et al., 2009). This highlights the importance of selecting isolates from multiple sources when determining variations in traits in populations and between species.

The temperatures studied in the immersed coil procedure included 63 and 72 °C, as these temperatures are the minimum used in the batch (63 °C for 30 min) and HTST (72 °C for 15 s) pasteurisation of milk, respectively. Two further temperatures above 72 °C, at 3 °C intervals, were selected for inclusion in D value determinations. The higher temperature of 81 °C was not used as none of the isolates was detected after the first heating interval. This situation also occurred for *E. faecalis* 2356p1 and *E. hirae* 2239p2 at 78 °C, so the additional temperature of 69 °C was assessed for these two isolates.

Two isolates (*E. faecalis* 2356p1, *E. hirae* 2239p2) had lower D values at both the lowest (63 °C) and higher temperatures compared to the other isolates tested. *E. hirae* 2452p2 also had a lower D value at 63 °C compared to the other isolates but showed greater heat resistance in general as indicated by the higher $z$ value. In contrast, two other isolates (*E. faecium* 2239p2, *E. hirae* 2239p2) exhibited greater heat resistance at 63 °C yet they were similar to the majority of isolates at the higher temperatures. This illustrates that heat resistance for particular isolates or species over a range of temperatures cannot necessarily be estimated from assessments at one or two temperatures.

Log$_{10}$ reductions were calculated from the pasteurisation data (63 °C/30 min) of Rao et al. (1986) and Shannon et al. (1970) for *Streptococcus faecalis* and its subspecies *lactis* and *zymogenes*. In contrast to the average log$_{10}$ reduction of 1.3 log$_{10}$ cfu/mL for *E. faecalis* (63 °C/30 min) in this study, the log$_{10}$ reductions in the above mentioned studies were 2.5 and 4.2 log$_{10}$ cfu/mL, respectively. The subspecies are now no longer recognised and are considered to be the only variation of *S. faecalis* (Schleifer, 1986), which has been renamed *E. faecalis* (Schleifer and Kilpper-Bälz, 1984). Additionally, Shannon et al. (1970) obtained a log$_{10}$ reduction of 0.8 log$_{10}$ cfu/mL for *Streptococcus durans* and indicated that it was the most heat resistant species from the isolates that they tested. This is comparable to the results from this study. Rao et al. (1986) obtained a log$_{10}$ reduction of 0.2 log$_{10}$ cfu/mL for *Streptococcus faecium*, which is considerably lower than the results presented here. However these researchers considered *S. durans* to be a subspecies of *S. faecium*, which would have

---

**Table 3**

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>D values (min)</th>
<th>z values (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>63 °C</td>
<td>69 °C</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>ATCC 19433</td>
<td>3.6 (0.97)</td>
<td>0.3 (0.97)</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>2350p1</td>
<td>49.0 (0.97)</td>
<td>NT</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>2356p1</td>
<td>18.4 (0.99)</td>
<td>1.3 (0.99)</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>2276p1</td>
<td>34.8 (0.99)</td>
<td>NT</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>2299p2</td>
<td>33.7 (0.99)</td>
<td>NT</td>
</tr>
<tr>
<td><em>E. durans</em></td>
<td>2128p1</td>
<td>29.0 (0.99)</td>
<td>NT</td>
</tr>
<tr>
<td><em>E. durans</em></td>
<td>2151p1</td>
<td>26.6 (0.99)</td>
<td>NT</td>
</tr>
<tr>
<td><em>E. hirae</em></td>
<td>2239p2</td>
<td>17.1 (0.97)</td>
<td>5.0 (0.98)</td>
</tr>
<tr>
<td><em>E. hirae</em></td>
<td>2452p2</td>
<td>15.1 (0.98)</td>
<td>NT</td>
</tr>
</tbody>
</table>

*Correlation coefficient (R) values in brackets.*

*NA = not applicable.*

---

**Table 4**

Predicted log$_{10}$ reduction of enterococci at 72 °C using Weibull and linear regression models.

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>Log$_{10}$ reduction (cfu/mL) in 15 s</th>
<th>Time (min) to obtain a 6 log$_{10}$ reduction using the Weibull model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Weibull</td>
<td>Linear regression</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>2350p1</td>
<td>0.58</td>
<td>0.09</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>2356p1</td>
<td>2.25</td>
<td>0.79</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>2276p1</td>
<td>0.52</td>
<td>0.05</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>2299p2</td>
<td>0.54</td>
<td>0.16</td>
</tr>
<tr>
<td><em>E. durans</em></td>
<td>2128p1</td>
<td>0.20</td>
<td>0.09</td>
</tr>
<tr>
<td><em>E. durans</em></td>
<td>2151p1</td>
<td>0.10</td>
<td>0.08</td>
</tr>
<tr>
<td><em>E. hirae</em></td>
<td>2239p2</td>
<td>0.23</td>
<td>0.19</td>
</tr>
<tr>
<td><em>E. hirae</em></td>
<td>2452p2</td>
<td>0.30</td>
<td>0.05</td>
</tr>
</tbody>
</table>
affected any results attributable to species. E. hirae was first described in 1984 and isolates were possibly assigned to either S. faecium or S. durans previously (Devriese and Pot, 1995). The heat resistance of E. hirae has been infrequently characterised but one study found that it had a lower thermal resistance than E. faecium (Renner and Peters, 1999). In that study, E. faecium ATCC 6057 had a D value of 4.5 min at 65 °C whereas E. hirae ATCC 10541 had a D value of 1.5 min at 65 °C. Only one isolate of each species was assessed. The contrasting results for E. hirae in the current study highlight the importance of assessing multiple isolates from different sources for the characterisation of traits attributable to species.

Earlier studies on heat resistance employed methods that took from 20 s to 5 min for the test sample to reach the designated temperature (Shannon et al., 1970), which is not ideal when taking measurements at 15 s where any errors in the come-up time could be a significant portion of the test period. Some methods involved the preparation of multiple sample ampoules (Ghazala et al., 1995; Tait et al., 1991), which were manually removed from waterbaths. The preparation of multiple ampoules is time consuming while the manual removal of samples in several second intervals can introduce timing errors. The use of an immersed coil apparatus, such as the one employed in this study, overcomes these problems (Cole and Jones, 1990) by requiring the preparation of only one bulk sample, which is delivered at precise times and in precise volumes.

Comparison of the D values obtained in this study with those of other studies is difficult as the procedures, heating menstrua, and temperatures vary. The procedure used in the present study, using a submersed, heated coil will more closely resemble the situation in industry than previous laboratory methods. Also, several of the temperatures used in this study, 69, 72 and 78 °C, have not been reported in other work. The D values obtained for the Type strain E. faecalis ATCC 19433 in the current study are consistent with the logarithmic reductions obtained for this strain in nutrient broth at 65 and 71 °C in the study by Bradley and Fraise (1996), and with D values obtained in Sorenson’s buffer (Magnus et al., 1986). In a trial using 3.3-Dimethylglutaric acid-sodium hydroxide buffer at pH 7 as the heated menstruum, E. faecalis had a D value of 1.3 min at 63 °C (Tait et al., 1991), which is considerably lower than the D values of 18.4 and 49.0 min of E. faecalis in BHI broth in this study. BHI broth has been recommended for use in thermal resistance studies since it has a higher thermal resistance than buffer and it more closely resembles a food system (Simpson et al., 1994). On the other hand, the use of unbuffered BHI as a growth medium could elicit an acid adaptation response that increases heat resistance, as has been shown with E. faecium (Fernández et al., 2009). In a study by Batish et al. (1988), BHI broth was compared with full cream milk. At 63 °C, D values of E. faecalis were 7 to 17.5 min in full cream milk and 4.5 to 7.5 min in BHI broth, while D values of E. faecium were 25.5 and 9 min in milk and broth, respectively. That study also found that increased milk fat influenced thermal resistance. Similarly, the heat resistance of E. faecalis was greater in whole milk than in a saline solution (Aguirre et al., 2009). This differs from other reports in which increased milk fat did not increase heat resistance (Kornacki and Marth, 1992; Shannon et al., 1970). Thermal resistance of E. faecium was increased in ground beef when the fat content was increased from 4% to 12%, but this effect only occurred at lower heat treatments (58–65 °C) and fat content was not a significant factor at the higher temperature of 68 °C (Ma et al., 2007). Milk was not used as the heating menstruum in the present study since the potential for fouling and blocking the small diameter heated coil during the long holding times at the lower treatment temperatures could have been problematic. However based on reported differences between BHI and milk, some comparative testing to confirm or refute previous differences is warranted.

The other temperature common to this and other heat resistance studies was 75 °C. At this temperature, an isolate of E. faecium isolated from ham had a D value of 0.6 min in cooked meat broth (Ghazala et al., 1995) which was similar to the E. faecium isolate with median heat resistance in this study. E. durans ATCC 19432 had D values of 0.6 and 0.8 in BHI broth (Simpson et al., 1994), although that study used selective media (KF Streptococcus agar and M-Enterococcus agar) to obtain post-heat treatment counts. Selective media may not be the best recovery media for bacteria that have undergone a stressful treatment, as some selective agents in the media may prevent recovery of injured cells. However, the use of selective media may be unavoidable in situations where there is a mixed bacterial population. The longer plate incubation time of 72 h required in the current study to obtain maximum survival recovery suggests that damage has occurred to the cells during the heating process that has necessitated additional recovery time compared with the typical 24 h incubation of healthy cells.

Comparisons of the z values of E. faecalis, E. faecium and E. durans could be made with other published studies. In the present study, E. durans had z values of 8.7 and 8.8 °C, which was slightly less than the z values of 9.5–9.7 °C found in another study using BHI broth as the heating menstruum (Simpson et al., 1994). Other researchers have obtained z values of 5.2 °C for E. faecalis (Tait et al., 1991) and 4.5 °C for E. faecium (Martinez et al., 2003) in various buffers. When species obtained from cheese were compared for heat resistance in buffer, within one study, z values were 2.5–3.2 °C for E. faecalis, 3.6 °C for E. faecium and 4.2 °C for E. durans (Sanz Perez et al., 1982). Although E. durans showed the greatest heat resistance, these values are all quite low compared with the results in this study. This may reflect the effects of growth and survival in different environments as the isolates in this study were obtained from laboratory pasteurised milk and therefore already possessed some heat resistance compared with other enterococci. Accordingly, enterococci isolated from pasteurised ham have been shown to have higher z values, with E. faecalis and E. faecium having z values of 7.2–8.1 °C and 8.1–8.3 °C in buffer, respectively (Magnus et al., 1986).

A factor, which was not investigated in this study, was the effect that initial growth temperature may have on the heat resistance of the cultures. For this study, cultures were initially grown at 37 °C, close to their optimum temperature. Martinez et al. (2003) found that an E. faecium isolate from cheese was more heat resistant when the culture was grown at lower temperatures (5°C), particularly when the growth period was extended. Growth at 5°C would more closely reflect the situation in the dairy industry, where milk is stored at approximately 4 °C or slightly higher if proper storage conditions have not been maintained. However, this other work only investigated thermal inactivation at 70 °C and the cultures were not chilled after heat treatment. The current Australian Food Standards Code for the processing of milk requires that pasteurisation be conducted for a minimum of 15 s at 72 °C, followed by immediate shock cooling to 4.5 °C (FSANZ, 2004). Cold shock can affect the recovery of enterococci (Hansen and Riemann, 1963) so it is an important step in the process. In the current study, cultures were immediately cooled after heat treatment as this represents a condition of pasteurisation in the dairy industry. Further studies combining lower culture growth temperatures prior to pasteurisation, minimum 72 °C inactivation temperatures and post-treatment cooling would provide further information. Interestingly, Martinez et al. (2003) found that the phase of growth and the growth temperature of the cultures did not significantly (p > 0.05) affect z values for E. faecium. If this follows true for other species, then the z values obtained in the current study would not be different had the isolates been grown at 4 °C instead of 37 °C.

Both of the E. hirae curves at 63 °C exhibited shoulders, though this was not as noticeable for the other isolates, possibly because the other isolates, with the exception of E. faecalis 2356p1, did not have as great a total reduction by the last sampling time. Shoulders can be due to the tendency of the culture to form clumps, pairs or chains (Magnus et al., 1986). E. hirae 2452p2 did form cohesive,
noticeable clumps in BHI broth, which required longer vortexing in order to minimise counting inconsistencies on agar plates. At the lowest temperature studied here, the holding time was extensive, which would have enabled heat penetration of any clumps present. When the higher temperatures were assessed, the holding times were very short and heat penetration may not have extended through entire clumps. This could lead to a residual population inside the clump which had not received the full heat treatment and could thus survive and appear to be more resistant to heat inactivation. This tendency to clump may have provided E. hirae 2452p2 with a certain degree of protection at the higher temperatures, allowing it to survive longer than the other more miscible cultures.

Although industry has traditionally utilised D values as a measure of the heat resistance of microorganisms, they can have limited value when microorganisms have non-linear survival curves. Linear regression works well when the survival curves decrease at a constant rate over time. In reality, microorganisms may exhibit shoulders at the beginning or tailing at the end of survival curves. Linear analysis does not fit well to such curves. This is indicated, in part, by lower correlation coefficient (R) values than for the more linear curves. At higher temperatures, culture numbers could have a rapid initial drop, followed by a gradual tailing. Other studies have found shoulders at the beginning of E. faecium (Ghazala et al., 1995; Magnus et al., 1988) and E. faecalis (Magnus et al., 1988) survivor curves and variable tailing at the end of E. faecium survival curves (Ross et al., 1998). The tailing phenomena may be due to the presence of unequal heat resistance throughout the bacterial population (Hansen and Riemann, 1963). If D values are calculated from the slope generated by the initial rapid drop in microbial numbers, the D values would underestimate the number of survivors that may exist within a population. Likewise, if the D values are calculated from the slope following an initial shoulder, the number of survivors would be overestimated. Therefore it is important that both the shoulder and the tail end of curve are taken into consideration. The only accurate way to estimate heat resistance is with a fitted curve. For a more precise estimation of thermal death, the Weibull model has been used to successfully fit lines to non-linear survival curves (Mattick et al., 2001) and was used to fit curves to the present data. The model estimated that pasteurisation conditions produced only a 0.1 to 2.25 log_{10} cfu/mL reduction and that the time required to obtain a 6 log_{10} cfu/mL reduction at 72 °C was between 6 and 34 min for the more thermolabile enterococci found in dairy products. Since a pasteurisation time of several minutes is not feasible for industry, this indicates that a higher temperature is required if the more heat resistant enterococci are to be eliminated from raw milk. The presence of enterococci in milk post-pasteurisation could pose a food spoilage problem, as enterococci from ewes' cheese and milk have been shown to have lipoase activity (Senedto et al., 2003).

Acknowledgements

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The authors would also like to thank Ms Marie Collier for dedicated technical support and Dr Cindy Stewart (formerly with CSIRO Division of Food and Nutritional Sciences, Sydney) for her comments and assistance with the Weibull model.

References


Appendix C - List of publications, conference posters, presentations and reports prepared as part of PhD candidature

Publications


Internet Publication

Note – this is no longer on the internet.

Conference Paper

Conference Posters


Oral Presentations


Reports

McAuley, C.M. and Craven, H.M. (2008) Report to determine if biofilm formation or susceptibility to sanitisers contributes to persistence of antibiotic-resistant enterococci and correlates with presence of antibiotic resistance genes. CSIRO funding report.


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Appendix D - List of additional publications, conference posters and presentations prepared during PhD candidature

Publications


**Conference Posters**


Walter, L., Ng, S., **McAuley, C.** and Buckow, R. (2013) Impact of Thermal and Pulsed Electric Field Processing on Microbial Stability of Raw Milk. *iFood2013*; 8-10 October 2013; Hannover, Germany. (Presented by Leonie Walter)


Oral Presentation

McAuley, C.M. (2013) Cronobacter and what we know in Australia, Australian Society for Microbiology Annual Scientific Meeting, 7-10 July 2013, Adelaide, Australia.
Appendix E - Maximum and minimum temperatures in Victoria

Supplementary Information published in McAuley et al., 2015

S1. Mean maximum temperatures for representative areas in the western, eastern and northern regions of Victoria.
Source: Bureau of Meteorology, Australia

![Graph of maximum temperatures]

S2. Mean minimum temperatures for representative areas in the western, eastern and northern regions of Victoria.
Source: Bureau of Meteorology, Australia

![Graph of minimum temperatures]
Appendix F - Autoclave times used at CSIRO Werribee

Autoclaving times used to autoclave liquid of various volumes in the Centenary Series (Atherton) autoclaves at Food Science Australia, Werribee. Autoclave times are based on achieving 121 °C for 15 min in the centre of the load and were calculated by Mr David Kerry.

<table>
<thead>
<tr>
<th>Volume range</th>
<th>Container type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flasks</td>
</tr>
<tr>
<td>From</td>
<td>To</td>
</tr>
<tr>
<td>1000 mL</td>
<td>2500 mL</td>
</tr>
<tr>
<td>500 mL</td>
<td>1000 mL</td>
</tr>
<tr>
<td>300 mL</td>
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<td>300 mL</td>
</tr>
<tr>
<td>0 mL</td>
<td>100 mL</td>
</tr>
</tbody>
</table>
## Appendix G - ANOVA tables for the data analysis of raw milk

Supplementary Information published in McAuley et al., 2015

**S3.** This ANOVA for the log$_{10}$ cfu/mL raw milk enterococci counts looks at the season and region effects and tests these against variation between factories and sampling time, respectively.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.(m.v.)</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F</th>
<th>pr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date stratum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Season</td>
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<td>12.1662</td>
<td>4.0554</td>
<td>8.23</td>
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</tr>
<tr>
<td>Residual</td>
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<td>3.9418</td>
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<td>0.33</td>
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<tr>
<td>Region.Factory stratum</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Region</td>
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<td>13.2730</td>
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<td></td>
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</tr>
<tr>
<td>Region.season</td>
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</tr>
<tr>
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<td>72.5627</td>
<td>1.5117</td>
<td>1.91</td>
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<td></td>
</tr>
<tr>
<td>Region.Factory. Sampling Time <em>Units</em> stratum</td>
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<td>140(4)</td>
<td>110.9496</td>
<td>0.7925</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>210(5)</td>
<td>222.3883</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**S4.** This ANOVA for the log$_{10}$ cfu/mL raw milk enterococci counts looks at the effects of factory and sampling time.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.(m.v.)</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F</th>
<th>pr.</th>
</tr>
</thead>
<tbody>
<tr>
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<td>16.8458</td>
<td>1.5314</td>
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<td>0.040</td>
<td></td>
</tr>
<tr>
<td>Factory</td>
<td>5</td>
<td>15.3435</td>
<td>3.0687</td>
<td>3.87</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Sampling Time.Factory</td>
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<td>1.91</td>
<td>0.001</td>
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</tr>
<tr>
<td>Residual</td>
<td>140(4)</td>
<td>110.9496</td>
<td>0.7925</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Total</td>
<td>210(5)</td>
<td>222.3883</td>
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Author/s:
McAuley, Catherine Mary

Title:
Studies on the prevalence, persistence and antibiotic resistance of enterococci from Australian dairy sources

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2016

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