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 thermotolerant 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 thermotolerant enterococci survived pasteurization in a small numbers of instances. All of the thermotolerant en-

terococci 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. Thermotolerant 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 Serron, 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).

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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 regionality, 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 thermotolerant 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 (10^0 to 10^{-2} dilutions) was spread onto CEBA, 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* 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 CEBA 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 CEBA plates to detect presumptive enterococci. The broths 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 cryobeads (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 $\log_{10}$ 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 ($\leq 5^\circ\text{C}$). 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 $\log_{10}$ 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 $\log_{10}$ 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 $\log_{10}$ cfu/mL), with counts ranging from <1 to 6.80 $\log_{10}$ cfu/mL (Figure 1) with an average of 2.48 $\log_{10}$ cfu/mL; most counts (77.3%) were <3 $\log_{10}$ 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 $\log_{10}$ cfu/mL).

The average counts of enterococci in raw milk from each factory throughout the year were all between 2 and 3 $\log_{10}$ 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 $\log_{10}$ 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.

Figure 2. Counts of enterococci in raw milk over the 4 seasons averaged across all of the factories. LSD = least significant difference.

E. malodoratus (0.6%), and *E. gallinarum*, *E. mundtii*, *E. pseudoavium*, *E. raffinosus*, *E. saccharolyticus*, and *E. sulfureus* (0.1%). Only 1 isolate each of the latter 6 species was found.

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 log₁₀ cfu/mL, respectively. The remaining 8 samples (16.6%) did not contain a predominant species. Four milk samples had enterococci counts of 5 log₁₀ 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. caseliflavus* 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 log₁₀ 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 log₁₀ cfu/mL) of approximately 4 log₁₀ 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 log₁₀ 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 log₁₀ 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 log₁₀ 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).

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.

Table 1. Detection of *Enterococcus* species and incidence of species type following factory pasteurization of milk and after 2 wk of incubation of milk samples at 4°C

Time after pasteurization (d)	No. of samples ¹ positive in the range (minimum cfu/mL detectable) ²					Incidence of species in samples (% of positive samples)
	ND ³	0.01	0.1	1	10	
0	19	5 ⁴	0	0	0	<i>E. durans</i> (80%) <i>E. malodoratus</i> (20%)
14	13	5	2	1	1 ⁵	<i>E. sulfureus</i> (44.4%) <i>E. asini</i> (33.3%) ⁶ <i>E. durans</i> (33.3%) ⁷ <i>E. hirae</i> (22.2%) <i>E. faecalis</i> (11.1%)

¹Twenty-four samples of pasteurized bulk milk were collected across season from factory B for plate counts; 22 of these were enriched to detect enterococci immediately after collection and then incubated for 2 wk at 4°C before retesting by plate counts and enrichment.

²Minimum number of cells/mL detectable by enriching of 100, 10, or 1 mL of pasteurized milk in Chromocult Enterococci broth (CEB; Merck, Darmstadt, Germany) or by direct plating of 0.1 mL on spread plates of CEBA (CEB with 15 g/L of Bacteriological Agar No. 1; Oxoid, Basingstoke, UK).

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

⁴Three *E. durans* in autumn samples, 1 *E. durans* in summer, and 1 *E. malodoratus* in winter.

⁵CEBA spread plate count average 2.82 log₁₀ cfu/mL.

⁶*E. asini* detected in 1- and 10-mL enrichments.

⁷*E. durans* detected in 0.1-mL spread plates.

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

Figure 4. Growth of *Enterococcus* species at 4 (solid lines) and 7°C (dashed lines) in inoculated milk: *E. faecalis* (○), *E. faecium* (□), *E. hirae* (Δ), and *E. durans* (◇).

(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 log₁₀ 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 log₁₀ 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 thermotolerant 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 transmis-

sion 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-

Table 2. Parameters of *Enterococcus* spp. growth curves at 4 and 7°C in milk determined from DMFit models (<http://modelling.combase.cc/membership/ComBaseLogin.aspx>)

Species	Incubation temperature (°C)	Initial value ¹ (log ₁₀ cfu/mL)	Final value (log ₁₀ cfu/mL)	Maximum rate ² (log ₁₀ cfu/mL per hour) × 100	Generation time (h)
<i>E. faecalis</i>	4	1.55	5.92	0.75	39.8
<i>E. faecium</i>		1.68	6.42	0.73	41.0
<i>E. hirae</i>		1.48	7.10	0.79	37.6
<i>E. durans</i>		1.47	6.76	0.85	35.5
<i>E. faecalis</i>	7	1.75	7.41	1.91	15.9
<i>E. faecium</i>		1.82	8.10	1.48	20.8
<i>E. hirae</i>		1.96	7.67	1.37	22.0
<i>E. durans</i>		1.51	8.01	1.65	19.5

¹Inoculated level.

²All 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 regionality. 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 thermophilic 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 log₁₀ 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 semi-quantitative. 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 10² 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.

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