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Heat resistance of thermoduric enterococci isolated from milk

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ABSTRACT

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 $\log_{10}$ reductions incurred after pasteurisation (63 °C/30 min) and ranked from highest to lowest $\log_{10}$ 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.

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). 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 conjugative 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 inactivate 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

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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 (Merck, Darmstadt, Germany) to which 15 g/L of Bacteriological Agar No. 1 (Oxoid, Basingstoke, Hampshire, England) was added. 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 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 E'toile, 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 pour 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.

Table 1
Enterococci isolates used in thermal death determinations.

Isolate	Species	Factory
ATCC 19433	*E. faecalis*	N/A
2350p1	*E. faecalis*	A
2356p1	*E. faecalis*	F
2276p1	*E. faecium*	C
2299p2	*E. faecium*	F
2128p1	*E. durans*	D
2151p1	*E. durans*	B
2239p2	*E. hirae*	A
2452p2	*E. hirae*	E

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, 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 ($LogS = -b*t\hat{}n$) 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). 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}