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The resistance to heat of thermo-resistant streptococci attached to stainless steel in the presence of milk

S Flint[1], J Brooks[2], P Bremer[3], K Walker[1] and E Hausman[1]

[1]*New Zealand Dairy Research Institute, Private Bag 11029, Palmerston North, New Zealand;* [2]*Institute of Food, Nutrition and Human Health, Massey University, Private Bag 11222, Palmerston North, New Zealand;* [3]*Department of Food Science, New Zealand Institute of Crop and Food Research, University of Otago, PO Box 56, Dunedin, New Zealand*

Skim milk residues had a significant impact on the sensitivity to heat of a dairy isolate of the thermo-resistant, *Streptococcus thermophilus*. Cells of *S. thermophilus* (H) suspended in water or in milk had *D* values at 60°C of 2.0 and 14 min, respectively. Cells of *S. thermophilus* (H) attached to stainless steel in the presence of water or milk had *D* values at 60°C of 2.2 and 8.1 min, respectively. The attached cells in both experiments were heat-treated in the presence of water. The increase in heat resistance could not be fully attributed to individual components (caseinate or whey) in the milk. The potential for thermo-resistant streptococci to survive heat treatment in a dairy manufacturing plant is therefore greater than may be expected for the organism in less complex environments.
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Introduction

Thermo-resistant streptococci are characterized by their ability to tolerate pasteurization (72°C, 15 s) and grow at temperatures up to 52°C [2]. Biofilms of thermo-resistant streptococci on the stainless steel surfaces of dairy manufacturing plants affect product quality because the bacteria slough into the product being processed [4].

Although it is recognized that under normal dairy processing conditions thermo-resistant streptococci survive heat treatments (pasteurization and cleaning), there are few scientific data on the specific tolerance of these organisms to heat and the impact of factors such as the bacterial strain, the suspending medium (e.g., skim milk) and the status of the cells (planktonic, attached or growing in a biofilm).

In this study, *D* values (the times required to reduce cell numbers by a factor of 10) were determined for a strain of *Streptococcus thermophilus* isolated from a dairy manufacturing plant. This bacterium was exposed to heat while in two different suspending media (water and skim milk) and in the various states in which these cells are believed to occur in dairy manufacturing plants: planktonic, attached and growing in a biofilm.

Materials and methods

Bacterial strains

S. thermophilus strain (H), the main organism used in this study, was isolated from pasteurized milk obtained from a cheese manufacturing plant. This organism was identified as *S. thermophilus* using the polymerase chain reaction (PCR) with primers specific for *S. thermophilus* [10]. The type strain *S. thermophilus* (ATCC 19258), an isolate from milk in a casein manufacturing plant, *S. thermophilus* (EF2) and a similar organism, *S. waius* (3/1), also from a casein manufacturing plant, were used to provide supporting information. For the routine preparation of cultures, the bacteria were cultured from frozen (-80°C) stock in M17 broth (Difco, Fort Richard Laboratories, Auckland, New Zealand). To obtain a suspended cell population, the bacteria were subcultured in M17 broth at 37°C for 18 h. The cells were centrifuged ($3000\times g$, 10 min, 4°C) and resuspended in either sterile deionised water or sterile skim milk.

Development of surface-associated populations

Attached cell populations were defined as cells that attached to stainless steel under conditions that did not allow for cell growth or replication. Attached cells were prepared by exposing stainless steel coupons (1 cm^2, 316L, 2 B surface finish) in a static system to cells from an 18-h culture of *S. thermophilus* (H) prepared in M17 medium and resuspended in either water (as a neutral medium containing negligible organic matter to act as a control) or skim milk. After 30 min of exposure at 22°C, the samples were removed and washed by swirling them in a test tube using five changes of sterile deionized water.

Biofilms of *S. thermophilus* (H) were prepared by placing stainless steel coupons, previously exposed to the bacteria to generate attached populations as described above, into silicone tubing in a continuously flowing reactor supplied with pasteurized skim milk at 37°C for 18 h.

Determining D values

To determine the sensitivity of either suspended or attached populations of thermo-resistant streptococci to heat, isolates were heat-treated in plastic WhirlPak bags (NASCO; Biolab Scientific, Palmerston North, New Zealand) in a water bath [1]. For thermal treatment, WhirlPak bags contained the samples (2 ml of liquid or a stainless steel coupon in 2 ml of water) to be tested. In addition, three WhirlPak bags containing samples were used to monitor the

Correspondence: Dr SH Flint, New Zealand Dairy Research Institute, Private Bag 11029, Palmerston North, New Zealand
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temperature. All were equilibrated in a water bath at 10°C to standardize the initial condition before being completely submerged below the water level in a preheated water bath at the temperature required for testing. To monitor the temperature profile of the sample during treatment, thermocouples (type t — copper/constantan) were placed in the centre of each of three replicate samples connected to a chart recorder (Yokogawa, Tokyo, Japan) that recorded temperatures at 3-s intervals. Zero time was taken as the time when at least two of the three samples reached the test temperature. A test sample was removed at time zero and placed in a cooled water bath at <10°C. The remaining test samples were removed at predetermined time intervals and placed in the cooled water bath. Upon completion of the thermal trials, all samples were removed from the cooled bath and cell numbers were estimated from cell viability determined using the Malthus microbiological growth analyser (Malthus Instruments, Stoke on Trent, UK) [6] or the BacTrac microbiological growth analyser (Sylab, Purkersdorf-Vienna, Austria). These growth analysers use impedance changes resulting from viable cell activity to estimate cell numbers.

Thermal death curves were prepared from the regression of the viable cell numbers against time for each heat treatment temperature (SigmaPlot; Jandel, San Rafael, CA, USA). *D* values were determined from the negative reciprocal of the slope of each thermal death curve. The *Z* values [change in temperature (°C) required to produce a 10-fold change in the *D* value] were determined from the negative reciprocal of the slope of each graph of $\log_{10} D$ values against temperature. Thermal death curves were prepared for: planktonic and attached cells of *S. thermophilus* (H) in water at 56, 58, 60, 62 and 64°C and 58, 60, 62, 64 and 66°C, respectively; planktonic cells in milk and cells attached in the presence of milk and then washed in deionized water at 58, 60, 62, 64 and 66°C; 18-h old biofilms prepared in a laboratory reactor at 60°C.

The coupons containing 18-h biofilms of *S. thermophilus* (H) grown in skim milk were washed five times in sterile deionized water before testing using the WhirlPak method described above at 60°C.

D values at 62°C were determined for *S. thermophilus* (ATCC 19258), *S. thermophilus* (EF2) and *S. waius* (3/1) attached to stainless steel in the presence of water or in the presence of milk, and then washed in deionized water before testing them. Heat treatment at 62°C was at an arbitrary temperature to provide data to support the trend observed with *S. thermophilus* (H).

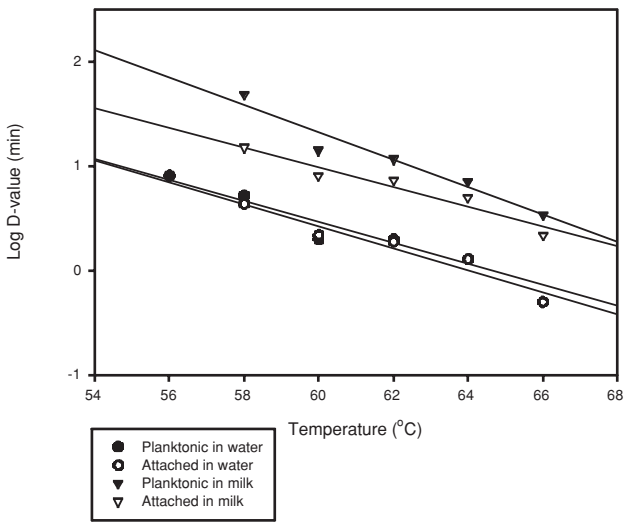

Figure 1 Regression of the $\log_{10} D$ values (min) for planktonic and attached cells of *S. thermophilus* (H) in milk and water.

Table 1 Summary of results for the heat treatment of *S. thermophilus* (H)

Cell status	*D* value (min) at 60°C	*Z* value
Planktonic in water	2.0	9.9
Planktonic in milk	14	7.6
Attached in water	2.2	9.4
Attached in milk	8.1	10.7
Biofilm	1.7	N.D.

N.D.=not done.

In an attempt to determine the milk components affecting the resistance to heat of cells attached to stainless steel, *S. thermophilus* (H) was attached to stainless steel in the presence of autoclaved solutions of whey, caseinate, combinations of whey and caseinate and lactose. *D* values were determined at 62°C using the WhirlPak bag method described above.

The statistical significance of the results was determined by covariate analysis using Minitab (Minitab; State College, Pennsylvania, USA). All experiments were done in duplicate. In addition, duplicate samples were taken at each data point for each experiment.

Results

D values from the thermal trials on planktonic and attached cells of *S. thermophilus* (H) in water and milk were plotted against temperature to evaluate the heat sensitivity of the cells (Figure 1). The best fit lines for the relationship *D versus* temperature using least squares regression are described by the following equations: planktonic cells in water, $D=-0.101(t)+6.53$, $r^2=0.92$; planktonic cells in milk, $D=-0.131(t)+9.18$, $r^2=0.92$; cells attached to stainless steel in the presence of water, $D=-0.106(t)+6.74$, $r^2=0.93$; and cells attached to stainless steel in the presence of milk, $D=-0.090(t)+6.66$, $r^2=0.93$, where $D=D$ value (min), t=temperature (°C) and r^2=regression value.

D values obtained for planktonic or attached cells were not significantly different (in water, planktonic $D=2.0$ min; attached $D=2.2$ min; $P>0.001$). However, the *D* values obtained for planktonic cells suspended in water or for cells attached to stainless steel in the presence of water were significantly less than the values obtained for cells suspended in milk or for cells attached to stainless steel in the presence of milk ($P<0.001$) (Table 1).

The *D* value of 1.7 min. ($r^2=0.95$) at 60°C for biofilms of *S. thermophilus* (H) grown in the presence of skim milk over 18 h was less than that for cells recently attached to stainless steel (Table 1).

The *D* values for two other strains of *S. thermophilus* and one strain of *S. waius* showed the same trend with cells attached to stainless steel in the presence of skim milk showing a greater

Table 2 *D* values at 62°C for *S. thermophilus* and *S. waius*

Strain	Attached in water (*D*)	Attached in milk (*D*)
S. thermophilus ATCC 19258	1.83	4.16
S. thermophilus EF2	0.50	3.34
S. waius 3/1	0.93	10.43

Table 3 The effect of milk components during attachment to stainless steel on the resistance to heat of *S. thermophilus* (H)

Attachment medium	*D* values (min) at 60°C
Water	0.82
Milk	5.43
Sodium caseinate	1.82
Whey	1.61
Sodium caseinate+whey	3.56
Lactose	0.94

resistance to heat than cells attached in the presence of water (Table 2).

Caseinate and whey reduced the effectiveness of heat on attached cells of *S. thermophilus* (H) but to a lesser extent than that observed with cells attached in the presence of skim milk. Combinations of caseinate and whey to the levels naturally found in milk had a greater effect on the resistance to heat than caseinate and whey individually (Table 3). Lactose had no effect on the resistance of *S. thermophilus* (H) to heat.

Discussion

The sensitivity of *S. thermophilus* (H) to heat varied depending on the environment. Planktonic cells in water or cells attached to stainless steel in the presence of water had similar heat sensitivities. However, cells attached in the presence of skim milk but heat-treated in the presence of water had an increased resistance to heat. Although the cells attached in the presence of milk were rinsed before heat treatment in water, residual milk protein on the cells or the substrate may protect the cells from heat. A similar reduction in the sensitivity to heat was seen with planktonic cells suspended in skim milk compared with water. Similar results were obtained for two other strains of *S. thermophilus* and one strain of *S. waius*. The increased resistance of cells on a stainless steel surface after exposure to milk proteins has not previously been reported. These results are, however, consistent with the effect of organic material (including milk and whey) in a suspension of bacteria on increasing the resistance of microorganisms to heat [8,11]. Both whey and caseinate had an individual and cumulative effect on the *D* value at 62°C. The cumulative effect of whey and caseinate did not equal that of the complete skim milk. This may be due to other factors in the milk contributing to protection of the organisms. Changes that occur to the milk proteins during the manufacture of whey protein concentrate and caseinate used in these trials may affect the natural ability of these components to protect attached cells from heat. The potential for thermo-resistant streptococci to survive heat treatment in a dairy manufacturing plant is therefore greater than may be expected for the organisms in other environments.

Biofilms of thermo-resistant streptococci grown for 18 h in skim milk were more sensitive to heat than cells that had recently attached to stainless steel in the presence of skim milk. The physiological state of cells in a biofilm is unique [5] and this could explain why cells within the biofilm behaved differently from cells that had recently attached to stainless steel. Our results demonstrate that the formation of a biofilm of *S. thermophilus* alone does not protect the bacteria from heat.

Cells within a biofilm have previously been reported to have an increased resistance to heat compared with planktonic cells. For *Listeria monocytogenes* [7], increased resistance to heat was associated with the amount of growth on the substrate; for *Salmonella enteritidis* [3], increased resistance to heat was believed to be due to a change in the physiology of the cell induced by attachment.

Our results demonstrate that environmental factors such as the medium (water or milk) may have a significant effect on the heat sensitivity of bacterial cells when attached to stainless steel. Organic molecules, such as proteins, readily attach to surfaces [9] and we hypothesize that, in our experiments, a complex of milk proteins on the substratum protected the bacterial cells from heat.

The sensitivity to heat of the *S. thermophilus* strain used in this trial provides evidence, from extrapolation of the *D* values, that the conditions of >70°C for 30 min, used in the routine cleaning of dairy manufacturing plant, are adequate to inactivate the cells in a biofilm. In practice, the ability to maintain this temperature for cleaning in a large plant may be difficult and a reduction in the temperature may enable survival. Accumulated organic material, which has not been removed over a succession of manufacturing runs, may provide additional protection to the cells, enhancing the resistance of the cells to heat.

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