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The growth of *Bacillus stearothermophilus* on stainless steel

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Aims: To determine the potential for *Bacillus stearothermophilus* cells to form biofilms of significance in dairy manufacture.

Methods and Results: The ability of isolates of *B. stearothermophilus* from dairy manufacturing plants to attach to stainless steel surfaces was demonstrated by exposing stainless steel samples to suspensions of spores or vegetative cells and determining the numbers attaching using impedance microbiology. Spores attached more readily than vegetative cells. The attachment of cells to stainless steel was increased 10–100-fold by the presence of milk fouling the stainless steel. The growth of *B. stearothermophilus* as a biofilm on stainless steel surfaces was determined using a continuously flowing experimental reactor. Vegetative cells were released in greater numbers than spores from biofilms of most strains studied. Biofilms of one strain (B11) were studied in detail. Biofilms of $> 10^6$ cells cm^{-2} formed in the reactor and released approximately 10^6 cells ml^{-1} into milk passing over the biofilm. A doubling time of 25 min was calculated for this organism grown as a biofilm.

Conclusions: The formation of biofilms of thermophilic *Bacillus* species within the plant appears to be a likely cause of contamination of manufactured dairy products. Methods to control the formation of biofilms in dairy manufacturing plants are required to reduce the contamination of dairy products with thermophilic bacilli.

Significance and Impact of the Study: Biofilms of *B. stearothermophilus* growing in dairy manufacturing plants can explain the contamination of dairy products with these bacteria.

INTRODUCTION

Bacillus stearothermophilus is a common contaminant of dairy products, particularly milk powder (Murphy *et al.* 1999). The organism is characterized by the ability of its spores to survive pasteurization (73 °C, 15 s) and grow at 65 °C. The organism is recognized as a problem in the manufacture of milk powder, as high levels of these bacteria may, after reconstitution of the milk powder, cause spoilage. The bacteria are present at low levels in raw milk, but may reach high levels in dairy products. This suggests that the bacteria grow during the manufacturing process.

The growth of thermophilic bacilli during the manufacture of milk powder is believed to occur as a biofilm. Biofilms are defined as the growth of micro-organisms and their extracellular polymeric material on a surface. Micro-organisms in a biofilm are generally more resistant than planktonic cells to cleaning chemicals and sanitizers commonly used to clean food manufacturing plants. This creates a potential problem as incompletely cleaned manufacturing plant surfaces may enable rapid biofilm growth during the subsequent manufacturing run. Bacteria can be released from the biofilm contaminating product flowing through the plant. This is known as the biotransfer potential of the biofilm.

The growth of vegetative cells of *B. stearothermophilus* on the surface of a milk powder manufacturing plant has been recorded (Stadhouders *et al.* 1982); however, this is one of the few articles published on biofilms of spore formers. Husmark and Rönner (1992) reported that spores generally attach to surfaces at a greater rate than vegetative cells, a process facilitated by their relatively high hydrophobicity. The effect of hydrophobicity on the adhesion of spores was demonstrated with two strains of *B. subtilis*, in which the spores differed in hydrophobicity, with the more hydrophobic strain attaching in greater numbers (Weincek *et al.*

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1991). Hodgkiss (1971) suggested that appendages on the spore surface promote adhesion but this is not always so (Husmark and Rönner 1992). The latter showed that spores of *B. stearothermophilus* are of low hydrophobicity (0–5%) and attach to stainless steel surfaces much less efficiently than the spores of some other *Bacillus* species. Langeveld *et al.* (1995) examined the adherence, growth and release of several bacteria, including *B. stearothermophilus*, in a tubular heat exchanger; however, little information was reported on this organism. In the present study, using several isolates, we examined some of the factors involved in the formation of a biofilm of *B. stearothermophilus*. The doubling times of the cells in the biofilm and the potential for releasing significant numbers of bacteria into the liquid flowing over the biofilm were determined.

MATERIALS AND METHODS

Origin and classification of the isolates

The following isolates of *B. stearothermophilus* were obtained from stock cultures held in a culture collection (Institute of Food Nutrition and Human Health, Massey University) originating from various sources, including five (marked*) from dairy manufacturing plants: B1*, B4*, B10*, B11, B13*, B16 (reference culture ATCC 7953), B17, B18 and 3A*.

Characterization of isolates

The identification of the isolates was confirmed by Gram stains, biochemical testing using the API 50CH test kit (bioMérieux, Montalieu Vercieu, France) and random amplified polymorphic DNA analysis using a random primer (OPR 13) (Ronimus *et al.* 1997).

Preparation of bacterial cells

Cultures were prepared in trypticase soy broth (BBL, Becton Dickinson, Cockeysville, MD, USA) at 55 °C for 18 h, washed twice by centrifugation (3000 *g*, 10 min) and resuspended in sterile deionized water.

Preparation of spores

To prepare spores, trypticase soy agar plates (TSA; BBL) were inoculated with 0·1 ml culture spread over the surface, incubated at 55 °C for 24–48 h and the growth washed from the plates with sterile distilled water. The recovered cells were washed in sterile distilled water and heat treated at 100 °C for 10 min to inactivate vegetative cells. The spore numbers were determined by plating serial dilutions of the heat-treated suspension on TSA and incubating at 55 °C for 24 h.

Attachment of bacteria to stainless steel

To determine the attachment to stainless steel, 1-cm^2 coupons of 316 stainless steel with a 2B surface finish, previously cleaned in 50% nitric acid and sterilized by autoclaving, were exposed to washed cells or spores (approximately 10^7 cells ml^{-1}) suspended in sterile deionized water. After 30 min at room temperature (22 °C), the coupons were washed with five changes of sterile deionized water and the number of cells attaching were determined by impedance (Flint *et al.* 1997) using a BacTrac impedance monitor (SyLab, MBH, Purkersdorf, Austria). Spores were differentiated from total bacterial cells by heat treating the coupons in a plastic bag ('Whirl Pak'; Nasco, Fort Atkinson, WI, USA) in a water-bath at 100 °C for 10 min. The number of viable spores was determined using impedance.

Experiments with milk foulant

To prepare a fouled surface, stainless steel coupons were immersed in pasteurized skim milk at pH 5·0 and autoclaved. In this way a visible foulant was produced on the surface of the stainless steel. The fouled coupons were exposed to a suspension of cells or spores for 30 min at room temperature. The fouled coupons were washed in five changes of sterile deionized water and the number of cells attaching determined using impedance.

Further experiments were performed using foulant produced in an experimental fouling rig designed at the New Zealand Dairy Research Institute, using milk heated by steam injection (Truong 1997). This foulant was subsequently sterilized by autoclaving. Wet foulant (10 g, equivalent to 0·2 g dry solids) was added to 10 ml cell suspension of *B. stearothermophilus* and allowed to stand at room temperature for 30 min. The foulant was removed and washed three times in 20 ml sterile water. The numbers of cells in the initial cell suspension, in the suspension following exposure to the foulant and in the foulant were determined by impedance.

Biofilm development

Biofilms were prepared in one of two reactors. The first was a continuously flowing laboratory reactor (Fig. 1) in which pasteurized skim milk, heated to 55 °C, was recirculated past 1-cm^2 coupons to which *B. stearothermophilus* was attached. Stainless steel coupons were inoculated as described previously and then inserted aseptically into the reactor tubing. Fresh skim milk was added and removed

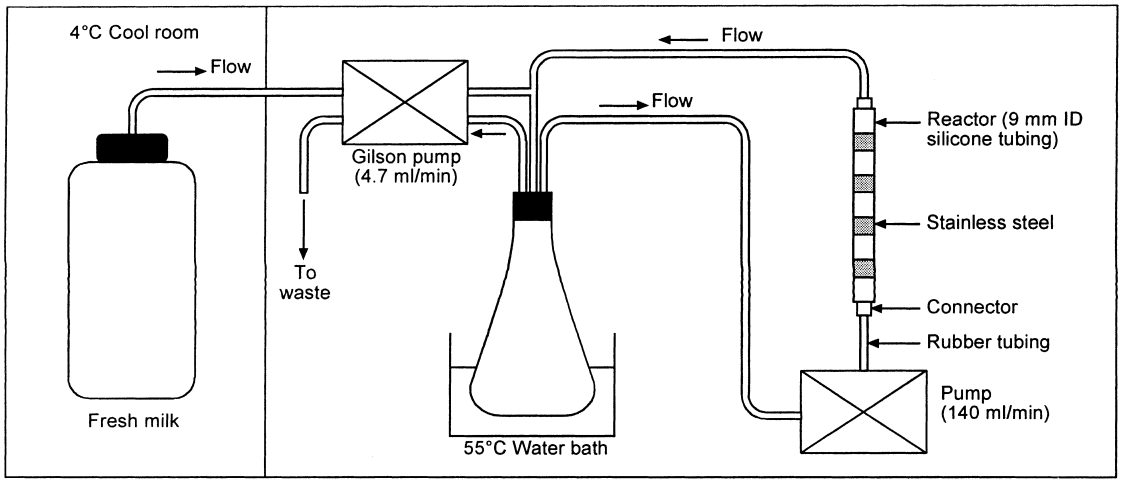

Fig. 1 Schematic diagram of the laboratory reactor 1

from the reactor at a rate determined by the doubling time of the bacteria (measured previously in a static culture) (i.e. half the total volume of skim milk in the reactor was replaced with fresh skim milk in the time taken for the numbers of bacteria to double). This ensured that there was no net growth in the skim milk that would affect the results. For some trials, skim milk was not recirculated through the reactor and was discarded after a single pass over the stainless steel coupons. This was to avoid any possibility of bacteria growing in the recirculating flask or other parts of the recirculation system that may influence the results.

A second reactor was designed to avoid the recirculation of milk and minimize the exposure to milk of surfaces other than the stainless steel under study. This second reactor consisted of a tube of 316L stainless steel with a 2B surface finish (total length 420 mm, internal diameter 4 mm) which consisted of five stainless steel sample pieces (length 35 mm, diameter 4 mm) (Fig. 2). The stainless steel sample pieces were connected together with silicone rubber

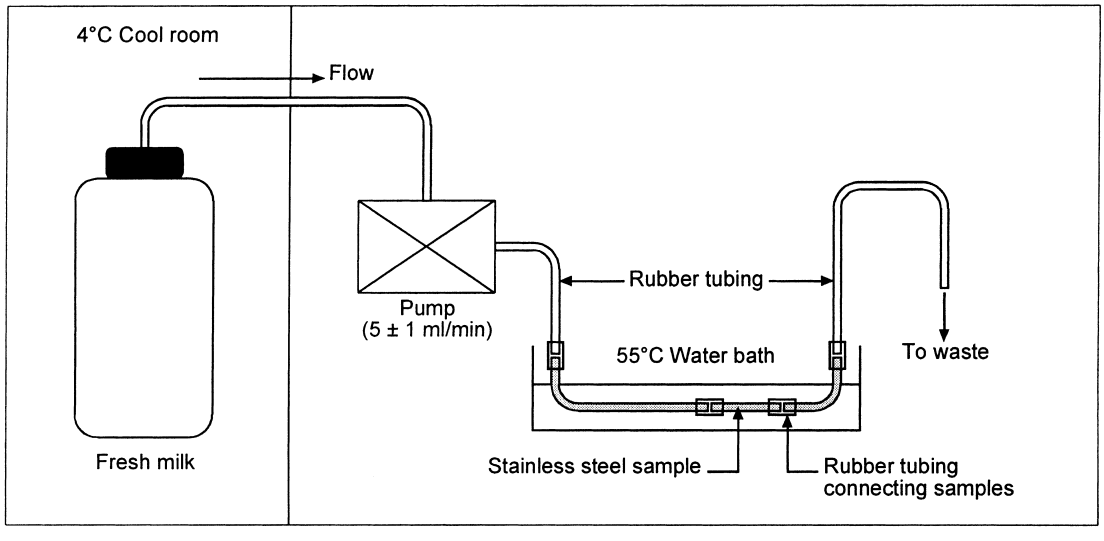

Fig. 2 Schematic diagram of the laboratory reactor 2 (only one sample piece shown)

tubing (Rubberware, Auckland, New Zealand) and the unit sterilized by autoclaving. The reactor tubing was inoculated by filling with an overnight culture of thermophilic *B. stearothermophilus* obtained from a laboratory stock. After 25 min at room temperature, the reactor tubing was washed once internally with sterile distilled water. By disconnecting one of the stainless steel samples after inoculation, the amount of inoculum could be measured for each reactor run.

The stainless steel reactor was immersed in a 55 °C water-bath, the optimum growth temperature for the thermophilic *Bacillus* species, to allow a biofilm to develop. The reactor tubing was connected to a peristaltic pump (Masterflex, Barant, Barrington, IL, USA), through which pasteurized skim milk stored at 4 °C was pumped at a flow rate of 5 ± 1 ml min^{-1}. The milk was pumped through the tubing over a period of 6 h. The milk was used only once; after flowing through the reactor the milk was discarded. The optimum growth temperature was reached once the milk had passed halfway down the reactor and samples were taken from this mid-point to the end of the reactor. After 2, 4, 5 and 6 h approximately, a stainless steel sample was taken in order to monitor biofilm growth on the stainless steel. This was accomplished by stopping the reactor pump, drying and disinfecting the outer surface of the reactor tube with 95% ethanol and then disconnecting one of the stainless steel samples and rubber tubes from the reactor. The outside surfaces of the stainless steel samples were swabbed with 5% formaldehyde solution, 95% ethanol and sterile distilled water before measuring the numbers of bacteria in the biofilm using impedance. The remaining sections of the reactor were reconnected and returned to the water-bath with minimal delay. The numbers of bacteria attached to the stainless steel surface were measured using impedance.

After 2 h, a sample of the milk entering the reactor ('milk in') was taken. After 2, 4, 5 and 6 h approximately, a sample of the milk passing out of the reactor ('milk out') was taken. The concentration of bacteria in the milk was measured using impedance.

Calculation of doubling times

The doubling times for the numbers of bacteria in the biofilms and in the 'milk out' were calculated with reference to Pirt (1975) using the following equations:

$$R = B_0 e^{k1t} + f\ F_0 \int e^{k2t}\ dt$$

$$= (\text{biofilm}) + (\text{flow})$$

where R = total number of bacteria produced by the reactor; B_0 = initial number of biofilm bacteria on the reactor surface; F_0 = initial number of bacteria in the milk flowing from the reactor; f = flow rate (ml min^{-1}); k1 = constant for biofilm cells; k2 = constant for cells in the flowing medium; t = time (min); specific growth rate (μ) = slope of the ln (biofilm + flow) *vs* time curve and doubling time $= \ln 2/\mu$.

RESULTS AND DISCUSSION

Attachment of spores to stainless steel

The preferential attachment of spores of *Bacillus* species has been reported previously (Husmark 1993). This was confirmed for two of the isolates tested with a greater proportion of spores than vegetative cells from inocula attaching to the stainless steel (Fig. 3). The percentage of spores in 1 ml suspension adhering varied with the isolate from 0·0025 to 0·25%.

Fouled surfaces attracted 10–100 times more vegetative cells and spores of *B. stearothermophilus* to the surface (two strains tested) than the clean stainless steel. This suggests that fouling may play a major role in the colonization of dairy manufacturing surfaces. However, the foulant produced on these experimental coupons may differ from that produced in a dairy manufacturing plant. This may have an effect on the structure of the fouling which may influence bacterial attachment.

Foulant in suspension also showed the ability to attract a large proportion of the cells of *B. stearothermophilus*. The

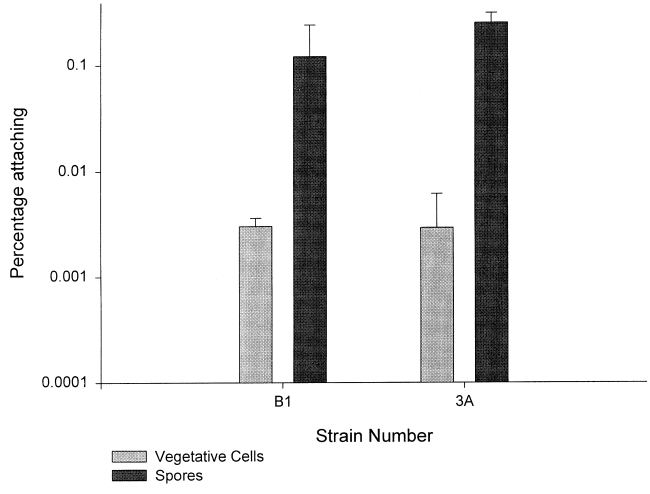

Fig. 3 Attachment of vegetative cells and spores of *Bacillus stearothermophilus* to clean stainless steel following 30 min exposure at room temperature (percentage of cells attached ± S.D.)

cell numbers in the cell suspension of 5×10^6 cells ml^{-1} were reduced by approximately 10-fold and therefore approximately 90% of the cells in a suspension were adsorbed to the foulant. Two factors are likely to be important here. Firstly, the biochemical nature of the foulant and secondly, the physical structure of the foulant are likely to interact with or trap the bacteria.

The effect of temperature on attachment

The total cells (spores and vegetative cells) of two strains of *B. stearothermophilus* (B11 and 3A) were exposed to clean stainless steel coupons at 20, 37 and 55 °C. The numbers attaching could not be related to the temperature with similar numbers of total cells attaching at each temperature (strain 3A, $7{\cdot}9 \times 10^4 \pm 2{\cdot}5 \times 10^4$; strain B11, $7{\cdot}9 \times 10^3 \pm 1{\cdot}5 \times 10^3$) cells cm^{-2}). This suggests that temperature is not an important factor in the attachment of spores.

Biofilm formation

To determine the ability of six different strains of *B. stearothermophilus* (B1, B4, B13, B16, B17 and 3A) to produce biofilms, coupons with attached spores were placed in a continuously flowing laboratory reactor (reactor 1) for 18 h. A clean reactor was used for each strain. The total number of viable cells and spores were determined in the biofilm using impedance (Fig. 4) and in the milk that had passed over the biofilm (Fig. 5).

There was some variation between the different strains of *B. stearothermophilus* in their ability to produce biofilms. After 6 h, two strains (B16 and B17) showed no increase in numbers colonizing the surface from the initial levels attaching (Fig. 4); however, bacteria were released into the milk (10^2–10^5 cells ml^{-1}) (Fig. 5). The biofilms of these two isolates consisted of low numbers of cells (approximately 100 cm^{-2}).

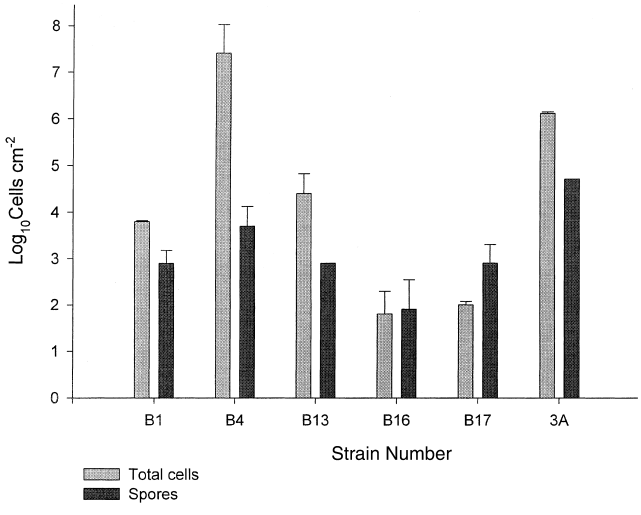

Fig. 4 Total viable cells (vegetative cells and spores) and spores in biofilms of six strains of *Bacillus stearothermophilus* (cell numbers ± S.D.)

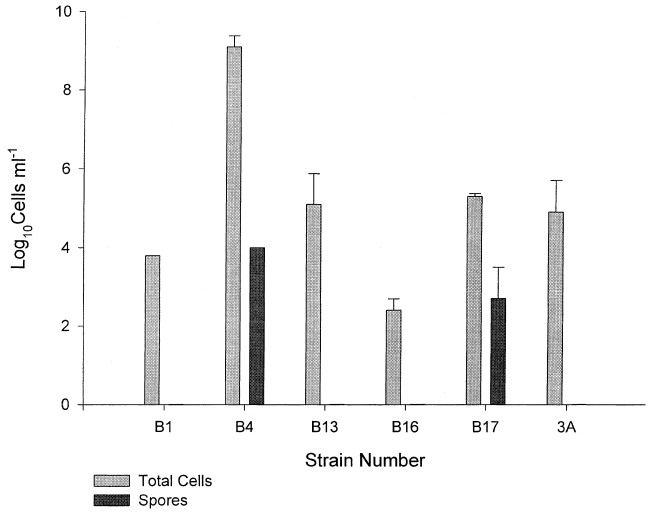

Fig. 5 Total viable cells (vegetative cells and spores) and spores released into flowing milk from biofilms of six strains of *Bacillus stearothermophilus* (cells ± S.D.)

The percentage of spores in the biofilms was variable. In biofilms where the total numbers of cells were $> 10^4$ cells cm^{-2}, the majority of cells in the biofilm were vegetative. Similarly, the majority of cells in the milk reservoir for the strains in which a good biofilm developed (B1, B4, B13 and 3A) were vegetative. The numbers of bacteria released into the milk ranged from $2{\cdot}4 \times 10^2$–$1{\cdot}3 \times 10^9$ cells ml^{-1}, indicating a significant biotransfer potential.

In reactor 1, the levels of bacteria, recorded in one experiment, on the silicone tubing (75 cells cm^{-2}), the stainless steel coupons ($3{\cdot}4 \times 10^2$ cells cm^{-2}) and in the milk ($4{\cdot}4 \times 10^5$ cells ml^{-1}) suggested that biofilms on the coupons were the most likely source of bacteria released into the milk.

To monitor the development of a biofilm and its biotransfer potential without the influence of recirculating milk, reactor 1 was modified to provide a flow system without recycle. The biofilm produced from inoculated coupons over 18 h contained fewer cells ($2{\cdot}7 \times 10^2$ cells cm^{-2}) than the total number of spores initially attached to the substrate ($1{\cdot}1 \times 10^4$ cells cm^{-2}) and consisted almost totally of spores or heat-resistant vegetative cells. The milk, with an initial count of < 10 cells ml^{-1}, contained $1{\cdot}8 \times 10^4$ cells ml^{-1}

after flowing through the system, representing a significant biotransfer potential. The experiment was repeated with the numbers of bacteria released into the milk flow rising from < 10 cells ml^{-1} initially to $5{\cdot}2 \times 10^3$ cells ml^{-1} after 5 h.

The above experiment was repeated with fouled coupons inoculated with spores of strain 3A ($1{\cdot}7 \times 10^7$ cells cm^{-2}). The numbers present in the milk increased faster than in previous experiments with $2{\cdot}5 \times 10^2$ cells ml^{-1} in the milk after 2 h incubation. The numbers of cells in the biofilm dropped by a factor of 27 from the initial inoculum to $4{\cdot}1 \times 10^5$ cells cm^{-2} containing 61 cells cm^{-2} spores or heat-resistant cells after 7 h incubation. The biotransfer potential was high with $1{\cdot}6 \times 10^7$ cells ml^{-1} released into the milk after 5 h incubation.

To determine the build up of biofilm on clean stainless steel and to attempt to reproduce the situation in a dairy manufacturing plant, the laboratory reactor was run with sterile coupons. After 18 h incubation, the thermophiles in the milk from the reactor were present at $1{\cdot}2 \times 10^6$ cells ml^{-1}, demonstrating significant growth originating from biofilms produced from the bacteria naturally present in pasteurized milk.

To confirm the observed biotransfer potentials in reactor 1, strain B11 was selected for three replicate trials using reactor 2. Although the reactors in all trials were inoculated in the same way, the amount of cells attached to the stainless steel tubing at the start of each experiment differed. The numbers ranged from $4{\cdot}0 \times 10^2$ to $2{\cdot}0 \times 10^5$ cells cm^{-2} on the total stainless steel surface of the reactor tubing (Fig. 6).

Biofilm growth was monitored over 6 h. Although the initial inoculum varied, the number of cells in the biofilm in the reactor after 6 h was similar, with maximum values of approximately $5{\cdot}0 \times 10^6$ cells cm^{-2}. The higher the number of inoculated cells the lower the net growth of the biofilm cells in the given time. These observations suggest a finite (optimal) density of biofilm cells under the conditions in this reactor system.

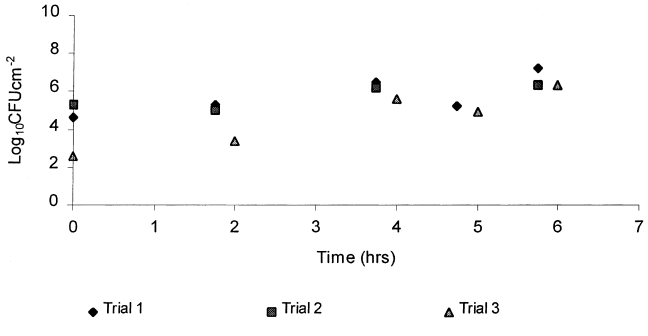

Fig. 6 Biofilm cell numbers (B11) on 1 cm^2 stainless steel in reactor 2 (expressed as cfu cm^{-2})

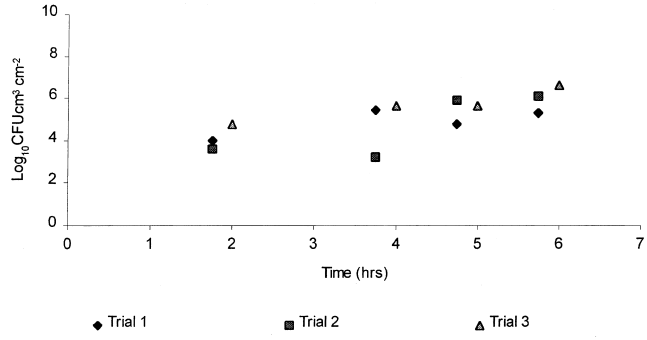

Fig. 7 Bacteria (B11) released ml^{-1} milk from 1 cm^2 stainless steel in reactor 2 (expressed as $\log_{10}$ cfu cm^{-3} cm^{-2})

The numbers of bacteria found in the 'milk out' are shown in Fig. 7. This shows a net increase in bacteria in the 'milk out' sample in each trial over a 6-h period with the number of cells increasing to similar levels in all reactors. Maximum values of approximately 1×10^6 cells ml^{-1} were reached. The concentration of cells in the 'milk in' sample (which varied from < 1 to 6×10^3 ml^{-1}) was subtracted from the concentration of cells in the 'milk out' to determine the net release of bacteria from the biofilm.

The amount of biofilm formed on exposed rubber tubing of the reactor was found to be negligible.

The doubling times calculated for strain B11 in the three type 2 reactors were approximately 25 min (range 21–32 min). This is longer than the doubling times of 15 min recorded for planktonic cells of thermophilic spore-forming bacteria (Basappa *et al.* 1974). This may be explained by the difficulty in identifying the logarithmic phase in the growth cycle with the limited number of samples taken from the reactor, resulting in all data being used in the calculations. The doubling time in similar experiments with another *B. stearothermophilus* strain (B13) was 19 min.

It is interesting to note that there are few reports of attempts to relate biotransfer potential to doubling time in biofilms. One publication (Langeveld *et al.* 1995) attempted to relate the observed biotransfer potential of bacteria to a theoretical calculation based on surface area and doubling time for bacteria in the plankonic state. Their measured results were significantly lower than the calculated results. This was explained by postulating the embedding of bacteria in denatured protein that prevented the release of bacteria into the milk.

CONCLUSIONS

These experiments demonstrate that *B. stearothermophilus* can form biofilms on clean stainless steel surfaces and release bacteria into milk. It appears that conditions in a biofilm in a laminar flow milk system are suitable for the growth of thermophilic spore-forming bacteria while the growth in milk as a culture medium is difficult. This may be due to a physiological change associated with biofilm colonization or to the supply of nutrients and removal of waste material by a flowing system enhancing the growth of these bacteria.

Attachment is the crucial first stage in the formation of a biofilm. Preferential attachment of spores was confirmed and artificially fouled surfaces attracted up to 100 times more cells than clean stainless steel surfaces.

For most strains tested, vegetative cells predominated both in 18 h biofilms and in the bacteria that were shed into milk passing over the biofilm. We hypothesize that the formation of biofilms of thermophilic *Bacillus* species involves the attachment of spores to the stainless steel surface which then germinate and release predominantly vegetative cells. Spores and vegetative cells that are released may attach to other surfaces and extend the growth of the biofilm.

The mechanism of attachment and the strength of attachment under different conditions need to be established. In particular, the effect of fouling, as found in industrial plants, requires further study and the role of spores in the development of a biofilm and the biotransfer potential need to be confirmed, particularly over different time periods. The growth of biofilms in different conditions (temperature, aeration, turbulent/laminar flow and different milk types) may vary and requires investigation. The survival of vegetative cells during the drying of milk powder needs to be established if contamination originating from biofilms is the primary source of thermophilic bacilli in milk powder.

There is no doubt that biofilms of thermophilic bacilli can develop rapidly to levels that cause significant contamination of milk passing the biofilm and therefore represent a source of contamination of dairy products.

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